CELLULAR INTERACTIONS IN THE HUMAN CORPUS LUTEUM

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DOCTOR OF MEDICINE

THE UNIVERSITY OF EDINBURGH

1998

DECLARATION

I hereby declare that the contents of this thesis were composed by myself, and that the work detailed in this thesis is my own, except where the contribution of others has been acknowledged in the text.

Faye Elizabeth Rodger

DEDICATION

I dedicate this thesis to my husband Angus for his patience and encouragement over the last three years, and also to my parents and grandparents for their love and support throughout my education.

ACKNOWLEDGEMENTS

I am indebted to the individuals listed below; without their invaluable assistance this thesis would not be complete.

I wish to thank Dr Peter Illingworth and Dr Hamish Fraser for their excellent supervision, stimulation and constructive criticism. Professor Alan McNeilly, Dr Rodney Kelly and Dr Hilary Critchley also provided constant support and enthusiasm. Thanks must also be give to Dr Steven Lunn, Dr Colin Duncan and Ms Fiona Young for helpful discussion, and to Mrs Gwen Cowen and Miss Pawlina Largue for instructing me in laboratory techniques. Invaluable advice on molecular biology was provided by Mr Joe Gaughan and Miss Joanne McVeigh, while immunohistochemical techniques benefited from the expertise and experience of Mr Mike Millar.

Donor recruitment was co-ordinated by Sister Vicki Reid-Thomas who also organised urinary LH and hCG treatment of donors.

Finally, I must thank the many women who donated the luteal tissue studied in this work, and the surgeons of the gynaecology department of the Royal Infirmary of Edinburgh for their assistance in obtaining luteal biopsies.

The work described in this thesis was funded by the Medical Research Council in the form of a Clinical Training Fellowship.

PUBLICATIONS RESULTING FROM THIS THESIS

Papers Published

Expression of the proto-oncogene bax does not vary with changing luteal function in women. (1998) **Rodger FE,** Fraser HM, Krajewski S, Reed JC and Illingworth PJ. Molecular Human Reproduction: 4, 27-32.

Immunolocalisation of the angiogenic growth factors VEGF and bFGF in the human corpus luteum throughout the luteal phase and in simulated early pregnancy. (1998) **Rodger FE,** Fraser HM, Young FM and Illingworth PJ. Submitted to Human Reproduction.

The human corpus luteum: reduction in macrophages during maternal recognition of pregnancy. (1998) Duncan WC, **Rodger FE** and Illingworth PJ. Human Reproduction: In Press.

Insulin-like growth factor binding protein-3 mRNA expression in endothelial cells of the monkey and human corpus luteum. (1998) Fraser HM, Lunn SF, Kim H, **Rodger FE**, Illingworth PJ and Erickson GF. European Society for Clinical Investigation, Abstracts of 32nd annual meeting.

Endothelial cell proliferation is triggered by the pre-ovulatory LH surge but not hCG stimulation in the human corpus luteum. (1997) **Rodger FE,** Young FM, Fraser HM and Illingworth PJ. Human Reproduction: 12, 1723-1729.

Distribution of leukocytes in the human corpus luteum throughout the luteal phase and in simulated early pregnancy. (1997) **Rodger FE,** Duncan WC, Largue PY, Fraser HM and Illingworth PJ. Human Reproduction: 12, Supplement 1, R228.

Endocrine control of the corpus luteum. (1997) Fraser HM, Duncan WC, Illingworth PJ, Lunn SF and **Rodger FE**. Human Reproduction: 12, Supplement 1, P061.

Endothelial cell distribution and proliferation throughout the luteal phase and in simulated early pregnancy. (1996) **Rodger FE**, Fraser HM, Young FM and Illingworth PJ. Biology of Reproduction: 54, Supplement 1, 243.

The effect of luteal rescue on LH receptor localisation in the human corpus luteum. (1996) Duncan WC, McNeilly AS, **Rodger FE** and Illingworth PJ. Journal of Reproduction and Fertility: Abstract series 15, 40.

Localisation of apoptosis after induced luteal regression in the primate. (1996) Young FM, **Rodger FE,** Cowen G, Woad K, Lunn S, Illingworth PJ and Fraser HM. Biology of Reproduction: 54, Supplement 1, 359.

Immunolocalisation of the proto-oncogene bcl-2 in the human corpus luteum. (1995) **Rodger FE**, Fraser HM, Duncan WC and Illingworth PJ. Human Reproduction: 10, 101-105.

Immunolocalisation of bcl-2 in the human corpus luteum. (1995) **Rodger FE**, Fraser HM and Illingworth PJ. Journal of Endocrinology: 144, Supplement, P328.

Measurement of dimeric inhibin-B throughout the human menstrual cycle. (1995) Groome NP, Illingworth PJ, O'Brien M, Pai R, **Rodger FE**, Mather J and McNeilly AS. Journal of Clinical Endocrinology and Metabolism: 81, 1401-1405.

PRESENTATIONS

Oral Presentations

Immunolocalisation of bcl-2 in the human corpus luteum. **Rodger FE,** Fraser HM, Duncan WC and Illingworth PJ. Munro Kerr Society, Edinburgh, February 1995.

Immunolocalisation of 17-alpha hydroxylase in the mare ovary. **Rodger FE,** Illingworth PJ, and Watson ED. Society for the Study of Fertility, Annual Conference, Dublin, July 1995.

Expression of the proto-oncogene bax in the human corpus luteum. **Rodger FE,** Fraser HM, Reed JC, Krajewski S and Illingworth PJ. National Ovarian Workshop, London, November 1995.

Endothelial proliferation is triggered by the preovulatory LH surge but not hCG stimulation in the human corpus luteum. **Rodger FE**, Fraser HM, Young FM and Illingworth PJ. Simpson Symposium number 9 (Reproductive Medicine) Edinburgh, August 1996.

The corpus luteum- growth or development? **Rodger FE**. Edinburgh Obstetrical Society, November 1996.

Expression of the angiogenic growth factors VEGF and bFGF in the human corpus luteum throughout the luteal phase and in simulated early pregnancy. **Rodger FE,** Fraser HM, Young FM and Illingworth PJ. National Ovarian Workshop, University of Bristol, April 1997.

Cellular interactions in the human corpus luteum. **Rodger FE.** Centre for Reproductive Biology, Edinburgh, November 1997.

Poster Presentations

Immunolocalisation of bcl-2 in the human corpus luteum. (1995) **Rodger FE**, Fraser HM and Illingworth PJ. British Endocrine Society, Annual Meeting, Warwick, March 1995.

Endothelial cell distribution and proliferation throughout the luteal phase and in simulated early pregnancy. (1996) **Rodger FE**, Fraser HM, Young FM and Illingworth PJ. Society for the Study of Reproduction, 28th Meeting, London, Ontario, July 1996.

Endothelial proliferation is triggered by the preovulatory LH surge but not hCG stimulation in the human corpus luteum. (1996) **Rodger FE**, Fraser HM, Young FM and Illingworth PJ. Simpson Symposium number 9. Edinburgh, August 1996.

ABBREVIATIONS

aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
FGF-1	fibroblast growth factor 1
FGF-2	fibroblast growth factor 2
cAMP	cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
eCG	equine chorionic gonadotrophin
FSH	follicle stimulating hormone
GAPDH	glucose phosphate dehydrogenase
GnRH	gonadotrophin releasing hormone
3-βHSD	3-β hydroxysteroid dehydrogenase
hCG	human chorionic gonadotrophin
HDL	high density lipoprotein
HRP	horseradish peroxidase
IL-1	interleukin-1
IL-2	interleukin-2
IL-8	interleukin-8
IgG	immunoglobulin-G
kDa	kilodaltons
LDL	low density lipoprotein
LH	luteinising hormone
MCP-1	monocyte chemoattractant protein-1
NBT	nitroblue tetrazium chloride
PCR	polymerase chain reaction
PGF2α	prostaglandin F2 alpha
PID	pelvic inflammatory disease
PMS	premenstrual syndrome
mRNA	messenger ribonucleic acid
ROS	reactive oxygen species
SDS-PAGE	sodiumdodecylsulphate polyacrylamide gel electrophoresis
SE	standard error
SOD	superoxide dismutase
TGF-β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumour necrotic factor alpha
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

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INTRODUCTION

1.1 Overview

The corpus luteum is a transient endocrine gland which is essential for the maintenance of early pregnancy in mammals. The gland persists to secrete progesterone in the presence of hCG in early pregnancy but will regress if conception has not occurred, allowing a new ovarian cycle to begin.

Control of the lifespan of the corpus luteum is essential for reproduction but is poorly understood. Neither the mechanisms which allow the corpus luteum to persist in early pregnancy nor those which limit the lifespan of the gland in non-conception cycles are known.

Many investigators have addressed these questions in recent years, and in vitro systems have been widely utilised to study luteal function. While studies examining luteinised granulosa cells have yielded important physiological data they provide a limited insight into the in vivo processes which may control luteal function.

In order to examine luteal physiology in vivo, a system has been designed to allow retrieval of human corpora lutea throughout closely monitored ovarian cycles. The system has also allowed retrieval of luteal tissue from women undergoing hormone treatment to simulate early pregnancy.

The experimental chapters of this thesis describe in detail the retrieval and characterisation of this tissue. Investigations utilizing the tissue to study potential control points in luteal maintenance and regression are then described.

The following introductory paragraphs set the scene for these investigations by describing what is already known about the structure, endocrinology and physiology of the corpus luteum in mammals, and in particular in women. Potential mediators of luteal maintenance and regression are discussed, with particular emphasis on areas examined in the experimental work of this thesis; the luteal vasculature and its growth, programmed cell death, and the immune system.

1.1.1 Historical background.

The corpus luteum was first described by Vesalius of Padua in 1555 and the secretory function of this tissue was acknowledged as early as 1609 when Fabricius described corpora lutea as 'multiple joined glands' (Auletta and Flint 1988). In the 17th century the importance of the corpus luteum for reproduction was first suggested by de Graaf who noted that in the rabbit the number of these ovarian glandular bodies corresponded to the number of embryos (de Graaf 1672). The term 'corpus luteum' was first used by Malphighi in 1697 (Auletta and Flint 1988). Not until 200 years later did the function of the corpus luteum come under further scrutiny, when it was proposed that the corpus luteum was a highly vascular gland of internal secretion (Prenant 1898). The first report of the biological significance of the gland was published in 1903 by Frankel who demonstrated that pregnancy was terminated in rabbits following lutectomy. The steroidogenic potential of the corpus luteum became clear in 1929 when Corner and Allen isolated progesterone from luteal tissue.

1.1.2 Luteal structure

General points

The corpus luteum forms from the dominant ovarian follicle after ovulation. The gland is a heterogeneous structure and is composed of different cell types including steroidogenic cells, endothelial cells, fibroblasts and leukocytes (Azmi

et al 1984; Lei et al 1991; Gillim et al 1969). The parenchyma of the corpus luteum exhibits infoldings around a central area of fibrin clot which develops in place of the previous follicular antrum. External to the parenchymal cells is a fibrous stroma which surrounds the gland and contains arterioles and venules, fibroblasts and immune cells (Corner 1956). Between the steroidogenic cells of the gland lie numerous small capillaries. The vascular bed of the corpus luteum is so dense that the majority of steroidogenic cells are in contact with at least one endothelial cell (Dharmarajan et al 1985; Zheng et al 1993; Reynolds et al 1992).

In addition to the cellular components of the corpus luteum, non-cellular components such as the extracellular matrix may be important not only for the structure but also for the function of the gland (Behrman et al 1993; Duncan et al 1996a;b;c).

Non-primate species

It is widely accepted that in ungulates the steroidogenic cells of the corpus luteum are derived from both theca and granulosa layers of the follicle (Rothchild 1981; Fritz and Speroff 1982). These layers may differentiate after ovulation to form small and large luteal cells (Auletta and Flint 1988). In domestic species large and small luteal cells do not occupy distinct compartments within the gland (Conley et al 1995).

Human

In women, structure of the corpus luteum is different to that seen in domestic species. The theca lutein layer lies at the periphery of the gland, with clumps of these small dense cells lying between the larger paler granulosa lutein cells and the fibrous stroma of the gland (Clement et al 1987). The theca lutein cells invaginate the granulosa lutein cell layer along fibrous trabeculae which give

the gland a characteristic wheel and spoke appearance (Sasano et al 1989; Corner 1956).

1.1.3 Luteal function

Production of steroid and protein hormones by the corpus luteum changes throughout the lifespan of the gland. Particular functional changes occur in the presence or absence of hCG in conception and non-conception cycles respectively.

Progesterone production

In the follicular phase of the cycle development of follicles is principally controlled by FSH. Under the influence of FSH, LH receptor expression is induced in the dominant follicle (Richards 1980; Segaloff et al 1990), and progesterone production in the human commences approximately 12 hours prior to the LH surge (Hoff et al 1983). Progesterone secretion increases as the corpus luteum forms, and reaches a maximum between 5 and 10 days after ovulation. If conception has not occurred, production of progesterone then falls from days 11-14 of the luteal phase facilitating menstruation and the start of a new ovarian cycle. If pregnancy has occurred, hCG secreted from the implanting fetoplacental unit permits continued secretion of the increasing amounts of luteal progesterone until the placenta assumes this function later in pregnancy (Strott et al 1969; Caldwell et al 1980; Illingworth et al 1990).

The human corpus luteum secretes up to 25mg of progesterone per day (Strauss et al 1981). Although the corpus luteum has the capability to synthesise cholesterol de novo (Talavera and Menon 1989), the majority of cholesterol is delivered to the gland by the circulation in the form of LDL (low density lipoproteins) and HDL (high density lipoproteins) (Tandeski et al 1996). The rate determining step for the conversion of cholesterol to progesterone is the transport of cholesterol into the mitochondria of the steroidogenic cells (Hall

1985; Behrman and Aten 1991). Recent advances have demonstrated that steroid acute regulatory protein (STAR) acts as a critical transport protein for cholesterol at this step, allowing for subsequent conversion of pregnenelone to progesterone by P450 enzymes on the inner mitochondrial membrane (Stocco and Clark 1996).

Oestrogen production

Corpora lutea of the majority of domestic species are not capable of oestrogen secretion (Conley et al 1995). In contrast, primate corpora lutea synthesise oestrogens in addition to progesterones (Ohara et al 1987; Hild-Petito et al 1989).

In primates, oestrogen may be an important regulator of ovarian function during the luteal phase. Luteal oestrogen feeds back at the level of the pituitary and hypothalamus to inhibit FSH and GnRH secretion and thus suppress follicle development during the luteal phase (Yamaji et al 1972; Bhattycharya et al 1972). In domestic species which do not produce luteal oestrogens FSH levels are not suppressed and follicles develop in waves throughout the luteal phase.

Inhibin production

In addition to steroid hormones, the corpus luteum also secretes protein hormones, principally inhibin and relaxin (Baird and Smith 1993). The primate corpus luteum produces inhibin A which may act through negative pituitary feedback in non-primate species (Campbell et al 1992), however experimental evidence is lacking for the speculation that a similar negative feedback loop exists in primates (Fraser and Lunn 1993; Fraser and Tsonis 1994). In parallel with progesterone, inhibin A is a marker of luteal function in women, and levels of inhibin decline after withdrawal of gonadotrophin support in the mid luteal phase (Fraser et al 1989) or enucleation of the corpus luteum in women and primates (Basset et al 1990; Illingworth et al 1991). The corpus luteum also

produces high levels of inhibin α- subunit which is thought to be biologically inactive. Its function may be paracrine but this has yet to be elucidated.

1.1.4 Changes associated with luteinisation

Follicles of primates and domestic species exhibit a clear demarcation between theca and granulosa layers. The layers are separated by a basement membrane and while the theca cell layer has a blood supply the granulosa lutein cell layer is avascular and is thought to obtain nutrients and oxygen by diffusion (Bassett 1943; McClure et al 1994). Morphological studies have demonstrated that following the LH surge the basement membrane between the granulosa and theca cells breaks down (McKay et al 1961) and the steroidogenic cells increase in size and number (Gillim et al 1969). Haemorrhage of blood into the central cavity and between the parenchymal cells of the gland is observed in the 48 hours following ovulation (Corner 1956). The central cavity later fills with fibrin and undergoes organisation (McKay et al 1961).

As these changes occur the vascular supply grows from the thecal cell compartment to supply the previously avascular granulosa cell compartment. Within 4 days of ovulation a dense vascular network has formed to supply the previously avascular granulosa cells with a rich vascular supply (Corner 1956; McClure et al 1994).

1.1.5 Changes associated with luteolysis

In a non-fertile cycle the corpus luteum undergoes both functional and structural regression. Endocrine activity diminishes at the end of the luteal phase (Baird 1984; Butcher et al 1974). The structure of the corpus luteum also starts to break down, diminishing in size and becoming less well demarcated from surrounding ovarian tissue (Deikman et al 1978; Braden et al 1988). During the

next ovarian cycle the corpus luteum becomes small and pale and is known as a corpus albicans.

Functional luteal regression.

From day 10 of the luteal phase onwards, the output of the human corpus luteum falls, in terms of progesterone, oestrogens and inhibin (Butcher et al 1974; Baird 1984). The fall in progesterone allows endometrial shedding and menstruation to occur while the corresponding the fall in oestrogen and inhibin permits upregulation of FSH and follicle recruitment (Knobil 1974).

Structural luteal regression.

This term refers to changes in the morphology of the corpus luteum at the end of its lifespan. The corpus luteum undergoes a reduction in size, and there is evidence to suggest that immune cell infiltration of the gland (Brannstrom and Norman 1993), vascular changes (Reynolds et al 1992; Redmer and Reynolds 1996) and cell death (Tilly 1996) may have major roles in this process.

Control of luteal lifespan

The control of luteal lifespan is subject to considerable species variation (Auletta and Flint 1988; Bazer 1992). If conception has not occurred in domestic species uterine PGF2α reaches the corpus luteum through a local counter current mechanism and induces regression (McCracken et al 1981). After hysterectomy in such animals the corpus luteum persists for a period of time equivalent to the gestational period of the species (Horton and Poyser 1976). In the absence of a uterine luteolytic signal, it appears that there is an inherent non-uterine regulator of luteal lifespan. It is possible that intra-ovarian factors induce luteal regression in these circumstances.

In women hysterectomy does not affect ovarian cyclicty (Beling et al 1970), and luteal regression is not induced by a uterine factor. The precise mediators of luteal regression in women are not well understood.

1.1.6 Changes associated with luteal rescue and pregnancy

In the presence of a conceptus, luteal function and structure are maintained, and increasing quantities of progesterone are secreted. The size and the vascular supply of the corpus luteum increase in line with these changes in hormone production (Atkinson et al 1975; Baber et al 1988; Salim et al 1994).

(a) Non-primates

In domestic animals such as sheep and cattle, trophoblast interferons exert a paracrine antiluteolytic effect on the endometrium to inhibit production of luteolytic pulses of PGF2α (Sharp et al 1989; Fincher et al 1986; Bazer 1992). In the absence of this luteolytic signal, luteal structure and function are maintained to support the developing intrauterine pregnancy. No definitive luteotropic factors have been described in domestic species.

(b) Human

Although there is no uterine luteolytic signal in the human, the uterus secretes a luteotropic factor, hCG, from the time of implantation (Hearn et al 1991). This glycoprotein binds to the common LH receptor to bring about the maintenance of function and structure which is termed luteal rescue. Progesterone and inhibin production are thus maintained in early pregnancy (Tovanabutra et al 1993). hCG is essential for luteal rescue as immunisation against this hormone results in decreased progesterone production and termination of pregnancy (Talwar 1997). The post-receptor mechanisms by which hCG brings about increased size and steroid production by the corpus luteum are unknown.

1.1.7 Clinical relevance

Progesterone secreted by the hCG rescued corpus luteum is essential for the maintenance of early pregnancy. Disruption of luteal function in early pregnancy, before or after implantation, is a possible means of post-coital contraception, monthly menses-inducing contraception or early termination of pregnancy.

Defects in luteal formation, rescue or secretion may lead to early miscarriage, a problem which affects 12 to 15% of pregnancies in the UK (Simpson 1996). However, no prospective randomised controlled trials exist to document therapeutic efficacy of supplementation of spontaneous pregnancy with exogenous progesterone (Carson 1996).

Improved understanding of the control of luteal function may provide a basis for the development of new forms of fertility control or interventions for early pregnancy loss.

1.1.8 Analogies with tumour tissue.

The corpus luteum has many features in common with neoplasms including an exceptionally quick growth (Zheng et al 1994), high blood flow (Bruce and Moor 1976), and high rates of proliferation (Jablonka-Shariff et al 1993) and angiogenesis (Reynolds et al 1992). In addition, in parallel with neoplastic tissue, growth of the corpus luteum may be regulated by programmed cell death (Tilly 1996), angiogenic factors (Koos 1989; Redmer and Reynolds 1996) and immune cells (Brannstrom and Norman 1993). There may be parallels between the physiological mechanisms important for formation and rescue of the corpus luteum and those involved in the growth of malignant tissue.

The low rates of malignant transformation in the corpus luteum (Jeffcoate 1987) may be related to the observation that steroidogenic luteal cells are fully differentiated. However, paracrine factors such as reducing agents or

angiogenesis inhibitors which may prevent cell growth in the corpus luteum may have therapeutic implications for malignancies in other tissues.

1.2 Lack of knowledge of factors which determine luteal lifespan.

1.2.1 LH/hCG and LH receptor

Luteal function is dependent on LH secretion from the pituitary (Smith 1930; Vande-Weil et al 1970; Hutchison and Zeleznik 1984). LH is a dimeric glycoprotein hormone secreted in pulses into the bloodstream which binds to G-protein coupled receptors on steroidogenic cells in the corpus luteum. Through these receptors it induces cAMP and progesterone synthesis (Dennefors et al 1982; McFarland et al 1989; Eyster et al 1985).

If LH secretion is blocked or removed then the corpus luteum undergoes regressive changes and progesterone secretion falls (Behrman et al 1971; Mais et al 1986). These changes are reversed by replacement of LH (Fraser et al 1985). In vitro, progesterone production from luteinised granulosa cells increases following LH stimulation (LeMaire et al 1968; Stouffer et al 1977). However secretion of LH remains at relatively low levels throughout the luteal phase and does not parallel changes in progesterone secretion (Behrman et al 1993). Spontaneous luteal regression in primates is not associated with a decrease in LH secretion (Behrman 1993). Neither is luteal regression due to changes in the pulsatile nature of LH secretion as decreased pulse frequency does not alter luteal function in primates (Zeleznik 1991). Other factors which interrupt luteal responsiveness to LH may thus be important.

Changes in the binding characteristics of the LH receptor have been implicated in luteal regression. LH binding studies in the corpus luteum show a fall in line with progesterone secretion during functional luteolysis but expression of the LH receptor is not down regulated at this time (Lee et al 1977; McNeilly et al 1980; Ravindranath et al 1992a). Changes in cAMP production have been

observed while LH receptor number is constant (Rojas et al 1989), and this may represent uncoupling of the LH receptor at the end of the luteal phase.

During early pregnancy hCG is essential for luteal rescue. LH binding and receptor expression remain unchanged at the time of luteal rescue (Duncan et al 1996d), however continued LH secretion is insufficient to prevent luteolysis (Behrman et al 1993). hCG therefore acts with the receptor in a manner distinct to that of LH. This is supported by the observation that the presence of persistently high levels of hCG are not associated with the down regulation of LH receptors which is observed with continuous secretion of LH (Caldwell et al 1980; Stouffer et al 1987).

1.2.2 Other putative factors in luteal regression

Factors implicated in luteal regression in non-primate species have been investigated in primate corpora lutea. In particular, the role of PGF2α in luteolysis in primates has been widely studied and is controversial because of a number of conflicting results.

Prostaglandins, including PGF2α, are synthesised in the primate corpus luteum (Challis et al 1976; Swanston et al 1977; Patwarden and Lanthier 1985; Houmard and Ottobre 1989). Although one group have found increased levels of PGF2α in regressing corpora lutea (Patwardhan and Lanthier 1985), other workers have not confirmed this finding (Michael et al 1994). Receptors for PGF2α are also present in primate corpus luteum (Powell et al 1975; Tanaka et al 1983), but it is not known if their abundance or distribution changes with luteal function.

In vitro, PGF2α reduces LH stimulated cAMP secretion in mid luteal phase tissue (Dennefors et al 1982; Khan-Dawood et al 1989), but in vivo studies give discordant results (Wentz and Jones 1973; Korda et al 1975; Bennegard et al 1991). Infusion of PGF2α into the corpus luteum of the rhesus monkey is luteolytic (Auletta et al 1984; Hearn and Webley 1987), but systemic,

intravaginal or intraovarian administration in women causes only a transient reduction in progesterone synthesis (Wentz and Jones 1973; Karim and Hillier 1979; Wilks 1980). The luteal phase is only slightly prolonged if prostaglandin synthesis is blocked by administration of ibuprofen, a cyclooxygenase inhibitor (Auletta and Flint 1988).

No studies have examined PGF2α or its receptor during luteal rescue in early pregnancy in women.

Oestrogens have also been implicated as physiological luteolysins in women. When given systemically or locally oestrogens are associated with a fall in progesterone production (Auletta et al 1972; Gore et al 1973; Karsch and Sutton 1976). This may however be due to inhibition of LH secretion due to feedback at the pituitary. If oestrogens are administered to primates by intraluteal pump at a rate that does not alter circulating levels of oestrogens or gonadotrophins luteal regression results (Auletta et al 1985). However further studies imply that this may not be the physiological process responsible for luteal regression as blocking studies using anti-oestrogens (Westphal and Resko 1982) or aromatase inhibitors (Ellinwood and Resko 1983) have failed to inhibit spontaneous luteal regression.

Other factors such as oxytocin (Tan et al 1982; Maas et al 1992) and free radicals (Margolin et al 1990; Endo et al 1993) have been implicated in luteal regression and maintenance in women. Despite a large body of literature luteolytic mechanisms in primates are poorly understood.

1.2.3 Interactions between luteal cell types may bring about changes in luteal function.

Previous studies have focused mainly on changes involving steroidogenic luteal cells, but the corpus luteum contains a greater number of non-steroidogenic than steroidogenic cells (Lei et al 1991; Zheng et al 1993). It is possible that the

various cell types in the gland may interact in a number of ways to bring about changes in luteal function and structure.

1.3 Proliferation in the corpus luteum

1.3.1 Luteal growth

As the progesterone output of the corpus luteum increases in both domestic species and primates during luteal formation and rescue, luteal weight also increases. Similarly, as progesterone secretion falls at the end of the cycle, luteal weight is reduced (Zheng et al 1994; Christenson and Stouffer 1996). These changes may be due to increases in the number or the size of the cells which constitute the corpus luteum.

1.3.2 Luteal cell proliferation

Changes in cell proliferation at different stages of luteal function have been studied using both morphological and immunohistochemical techniques. Morphological studies detect small numbers of mitotic figures in steroidogenic cells during the early luteal phase, but no mitoses are seen at later stages. (Corner 1956; Meyer and Bruce 1980; O'Shea et al 1980;1986). However, identification of morphological features of mitosis may not be the most sensitive or specific method of detecting proliferation.

Cells undergoing replication may only be identified by morphological features during the relatively short-lived M phase of the cell cycle, and pyknotic or fragmented nuclei may cause confusion in identifying mitotic bodies (Meyer 1982; Sasaki et al 1987). Furthermore, numbers of mitotic bodies may be altered by fixation techniques which can distort mitotic figures (Graem and Helweg-Larsen 1979; Cross et al 1990). These limitations have lead to utilisation of immunohistochemical proliferation markers in the study of luteal cell proliferation.

In corpora lutea of domestic species cells identified using immunohistochemical proliferation markers are most abundant during formation of the gland shortly after ovulation. With luteal maturation, proliferation rates decrease and remain at a constant level until the end of the cycle (Jablonka-Shariff et al 1993; Zheng et al 1994; Nicosia et al 1995). The majority of proliferating cells in the corpus luteum are of endothelial phenotype (Meyer and McGeachie 1988; Jablonka-Shariff et al 1993; Zheng et al 1994).

These morphological and immunohistochemical studies suggest that changes in progesterone production are associated with changing rates of luteal proliferation and angiogenesis in non-primates. At the outset of the work described in this thesis there was little information describing rates of proliferation in primate corpora lutea, throughout the luteal phase or during luteal rescue.

1.4 Luteal vasculature

1.4.1 Importance of luteal vasculature

The rapid growth and regression of the corpus luteum may be accompanied by equally rapid changes in its vascular bed and high rates of proliferation have been found in the endothelial cells of corpora lutea of domestic species (Meyer and McGeachie 1988; Jablonka-Shariff et al 1993; Zheng et al 1994). The corpus luteum is a highly vascular structure, with the highest blood supply of any tissue (Ellinwood et al 1978; Reynolds 1986; Reynolds et al 1992; 1994) and receives over 90% of the blood flow to the ovary (Bruce and Moor 1976; Bruce et al 1984). In the mature corpus luteum each steroidogenic cell is in direct contact with at least one endothelial cell (Dharmarajan et al 1985; Zheng et al 1993). The vascular bed of the corpus luteum is in the form of sinusoidal vessels which are not surrounded by smooth muscle. These are thought to offer low resistance to blood flow which may be dependant on arterial pressure and the extent of the vascular bed (Wiltbank et al 1990).

The vascular supply of the corpus luteum is essential for the delivery of gonadotrophic hormones, steroidogenic substrates and oxygen to steroidogenic cells (Koos 1989). Changes in the vascular supply of the corpus luteum may therefore influence its function.

1.4.2 Luteal blood flow in non-primates.

Serum progesterone is positively correlated to the rate of luteal blood flow in the ovary containing luteal tissue in non-primates (Nett et al 1976; Niswender et al 1976). A decrease in luteal blood flow is associated with ovine luteal regression, and blood flow increases in early pregnancy in rats (Bruce and Moor 1976; Bruce et al 1984). Blood flow to the rabbit corpus luteum is however unchanged by hCG treatment (Wiltbank et al 1989).

Care must be taken when extrapolating results of blood flow studies in domestic animals to primates, as PGF2α, the uterine luteolysin, may cause constriction of luteal vessels at regression (Pharris and Wyngarden 1969).

Ovarian blood flow in domestic species varies with changes in luteal function. Conversely, decreased vascularisation is associated with inadequate luteal phase in these species (Niswender and Nett 1988).

1.4.3 Clinical studies of luteal blood flow.

A number of studies have used Doppler ultrasound to examine luteal blood flow throughout the luteal phase in women. These studies have shown that luteal blood flow increases shortly after ovulation to a maximum level at days 7-9 after the LH peak (Bourne et al 1996; Alcazar et al 1996; Salim et al 1994). Blood flow to the corpus luteum then declines from luteal days 10 or 11 in a non fertile cycle. There is a positive correlation between systolic blood flow velocity, luteal volume and serum progesterone in women, and luteal low blood flow is associated with luteal phase defect in women (Glock et al 1995). No Doppler

ultrasound studies describe changes in blood flow over the first 14 days of normal pregnancy in women.

Clinical studies also provide evidence that luteal blood flow may be related to functional status in early pregnancy. These investigations have shown that a high resistive Doppler index (i.e. a low flow) is associated with missed abortion, incomplete and threatened miscarriage, and luteal phase defect (Deichert et al 1996; Salim et al 1994).

1.4.4 Angiogenesis

Morphological studies have shown that during development of the corpus luteum an intricate vascular network forms in a short space of time. The angiogenic process begins with formation of a new microcirculatory bed composed of arterioles, capillaries and venules. The sprouting of new blood vessels requires that endothelial cells degrade their surrounding extra cellular matrix, migrate towards an angiogenic stimulus and proliferate. Blood vessel formation is completed by formation of capillary lumina and differentiation of newly formed capillaries into arterioles and venules (Reynolds et al 1992).

1.5 Angiogenic factors

1.5.1 Luteal angiogenic factors

In domestic species the corpus luteum is characterised by a high rate of endothelial proliferation (Reynolds et al 1992), and blood vessel formation may regulate luteal function (Reynolds et al 1992;1994). The rate of vasculogenesis varies throughout the luteal phase, and is influenced by changes in LH secretion (Redmer et al 1988). There is controversy over expression of LH receptor by luteal endothelial cells (Ghinea et al 1994; Duncan et al 1996d) and paracrine factors may be involved in regulation of luteal angiogenesis. Luteal tissues, tissue extracts and conditioned media (Jakob et al 1977; Grazul-Bilska

et al 1992; Redmer et al 1985) have been shown to stimulate angiogenesis in vivo and in vitro bioassays.

1.5.2 Angiogenic potential of the corpus luteum.

Prenant noted that the corpus luteum was a highly vascular structure in 1898, but the angiogenic potential of the corpus luteum was not demonstrated until 79 years later. Studies implanting samples of luteal tissue into chick chorioallantoic membrane (an in vivo assay of angiogenic potential) showed that bovine luteal tissue could induce neovascularisaton (Jakob et al 1977, Redmer et al 1988). Previously this effect had been observed with implanted malignant tumour tissue but not with normal adult tissues (Folkman and Cotran 1976). Angiogenic activity was subsequently observed using luteal tissue from rabbits (Gospodarowicz and Thakral 1978), pigs (Heder et al 1979; Ricke et al 1995) and rats (Koos and Le Maire 1983), and endothelial migration was induced by ovine, bovine and porcine luteal extracts (Redmer et al 1988; Grazul- Bilska et al 1991; Doraiswamy et al 1995a;b). Subsequent studies demonstrated that the ovine corpus luteum induced proliferation and migration of endothelial cells at different stages of luteal function and in pregnancy (Grazul- Bilska et al 1992;1995).

Attempts to identify secreted angiogenic mediators in luteal extracts isolated a factor which bound strongly to heparin, was heat labile and was neutralised by antibodies directed against bFGF (basic fibroblast growth factor) (Grazul- Bilska et al 1993). A further secreted angiogenic factor was neutralised by antibodies directed against VEGF (vascular endothelial growth factor) (Doraiswamy et al 1995a). bFGF and VEGF may be principal local regulators of angiogenesis in the ovary (Koos 1989; Redmer and Reynolds 1996).

1.5.3 Basic fibroblast growth factor

bFGF (also known as FGF-2) is one of the most potently angiogenic members of a family of nine mitogens known as fibroblast growth factors (Folkman and Klagsburn 1987). bFGF was first isolated from pituitary (Gospodarowicz et al 1985) and is synthesised by macrophages, parenchymal cells of tumours and endothelial cells (Basilico and Moscatelli 1992; Kuwabara et al 1995). Four different molecular weight forms of bFGF have been identified; bFGF18, 22, 22.5 and 24 (Florkiewicz and Somer 1989), arising from differential transcription and post-translation modification of a single gene product (Bugler et al 1991; Florkiewicz et al 1991; Quarto et al 1991; Renko et al 1991). The 18kDa form is predominantly located in the cytoplasm, while the larger forms localise to the nucleus (Renko et al 1991). bFGF lacks a signal sequence (Abraham et al 1986) and the mechanism for its release into the extracellular milieu is unknown (Rifkin and Moscatelli 1989).

bFGF stimulates endothelial cell growth , migration and protease synthesis in vitro (Moscatelli et al 1988;1986; Mignatti et al 1989) and promotes formation of differentiated capillary tubes in vivo (Montesano et al 1986). bFGF is widely distributed throughout the body and is expressed in tissues where angiogenesis occurs at low as well as high rates (Gosparadowicz et al 1987; Folkman and Klagsburn 1987; Koos 1989; Basilico and Moscatelli 1992). This implies that bFGF may maintain the vasculature of certain tissues while inducing vascular growth in others.

This growth factor is not selective for endothelial cells and is mitogenic for a range of cells of mesodermal and neuroectodermal origin (Bikfalvi et al 1997). bFGF has been detected in bovine, ovine and porcine luteal tissues and conditioned media (Grazul-Bilska et al 1992; Zheng et al 1993; Schams et al 1994; Doraiswamy et al 1995b; Ricke et al 1995), and has been immunolocalised to steroidogenic cells and connective tissue tracts in ovine

and bovine corpora lutea (Grazul- Bilska et al 1992; Zheng et al 1993; Schams et al 1994). Expression of bFGF in the bovine ovary increases after formation of the corpus luteum to peak in the mid luteal phase and decline thereafter (Zheng et al 1993). In contrast, no variation in bFGF production has been observed at different stages in the ovine corpus luteum (Grazul- Bilska et al 1992). bFGF distribution or expression has not been investigated in the primate corpus luteum.

bFGF acts through a number of structurally related tyrosine kinase linked receptors (Erickson et al 1991; Zhu et al 1991) which are predominantly localised in endothelial cells in adult tissues (Bikfalvi 1997). FGF receptor-1 has been demonstrated in the rat in luteal endothelial cells at all stages and on steroidogenic cells prior to functional regression (Doraiswamy et al 1995b). FGF receptor-2 is present in steroidogenic cells throughout the lifespan of the gland (Redmer and Reynolds 1996).

1.5.4. Vascular endothelial growth factor

VEGF (also known as vascular permeability factor) is a 40kDa heparin binding glycoprotein dimer (Senger et al 1987; Connolly et al 1989b; Senger et al 1990; Ferrara and Henzel 1989; Gospodarowicz et al 1989). Four different isoforms arise from alternative splicing; VEGF 121, 165, 189 and 206, of which VEGF 165 is predominantly expressed (Tischer et al 1991; Park et al 1993). This growth factor has specific mitogenic effects on endothelial cells and acts as an angiogenic factor in vivo (Ferrara and Henzel 1989; Leung et al 1989), but also increases vascular permeability (Connolly et al 1989a;b; Senger et al 1993). Increased vascular permeability aids angiogenesis by causing leakage of plasma proteins and deposition of an extra-vascular fibrin gel which is a substrate for endothelial cell growth (Dvorak 1986; Dvorak et al 1987).

VEGF expression is upregulated by hypoxia in a similar manner to erythropoetin (Schweiki et al 1992; Minchenko et al 1994; Levy et al 1995;

Shima et al 1995), and VEGF and bFGF promote angiogenesis in a synergistic manner (Pepper et al 1992; Stavri et al 1995).

Distribution of VEGF mRNA is temporally and spatially related to proliferation of blood vessels in the rat (Phillips et al 1990), mouse (Schweiki et al 1993) and primate ovary (Ravindranath et al 1992b) and in the rat uterus (Cullinan-Bove and Koos 1993), suggesting that VEGF is a mediator of the cyclical growth of blood vessels that occurs in the female reproductive tract (Ferrara and Davis-Smith 1997). VEGF expression is also detectable around microvessels in areas where endothelial cells are normally quiescent such as kidney glomerulus, pituitary, heart, lung and brain (Brown et al 1992; Ferrara and Henzel 1989; Monacci et al 1993). It is possible that VEGF may be required not only to induce active vascular proliferation but also to maintain the differentiated state of blood vessels.

VEGF binds with high affinity to two tyrosine kinase linked receptors, flt and kdr (Shibuya et al 1990; Terman et al 1991;1992; de Vries et al 1992). Ligand autoradiography and in situ hybridisation studies in rat demonstrate that VEGF binding sites are largely restricted to vascular endothelium (Jakeman et al 1992; 1993; Peters et al 1993; Quinn et al 1993). VEGF and its receptors are vital for normal development. Inactivation of the VEGF gene in mice results in embryonic lethality in heterozygotes at days 11-12 of pregnancy (Carmeliet et al 1996; Ferrara et al 1996). This is due to gene dosage effect as VEGF mRNA can be detected at lower levels in heterozygous animals.

Mice homozygous for a targeted mutation in flt-1 die in utero between days 8.5 and 9.5 (Fong et al 1995). Inactivation of a murine form of kdr is associated with lethality at the same stage (Shalaby et al 1995).

At the outset of the work described in this thesis VEGF had been detected in cultures of luteinised human granulosa cells (Ravindranath et al 1992b) but had not been localised in the corpus luteum of any species.

VEGF expression may be influenced by the hypothalamic-pituitary-ovarian axis. VEGF expression increases in cultured bovine luteal cells in response to LH stimulation (Garrido et al 1993), and cultured human luteal cells upregulate VEGF production after hCG stimulation (Neulen et al 1995). In vivo studies reveal that continued treatment of monkeys with GnRH antagonist results in down regulation of luteal VEGF expression (Ravindranath et al 1992b). No studies have examined VEGF production in early pregnancy.

1.5.5 Other potential angiogenic factors

Although bFGF and VEGF are the best characterised angiogenic factors produced by the corpus luteum, other factors which affect blood vessel formation are produced by the gland.

The angiogenic activity of the ovine corpus luteum can be partly neutralised by antibodies directed against aFGF (acidic fibroblast growth factor), also known as FGF-1 (Grazul-Bilska et al 1993). This factor is a member of the same cytokine family as bFGF, but is a less potent endothelial mitogen (Folkman and Klagsburn 1987; Zagzag 1995). aFGF has been localised in large and small bovine luteal cells, although it is present at lower levels than bFGF (Zheng et al 1993).

Cytokines produced from immune and steroidogenic cell compartments also exert mitogenic effects on endothelial cells. In addition to functioning as chemotactic factors for immune cells, MCP-1 (monocyte chemoattractant protein-1) and IL-8 (interleukin 8) have angiogenic properties (Kelly et al 1997; Koch et al 1992; Nakashima et al 1995). These cytokines have been localised to steroidogenic cells in rat and pig corpora lutea (MCP-1) (Hosang et al 1994; Townson et al 1995) and luteinised granulosa cells in women (IL-8) (Arici et al 1986). MCP-1 and IL-8 may also be produced by immune cells in the corpus luteum (Arici et al 1996; Yoshimura et al 1987).

Macrophages are the most prevalent type of immune cell in the human corpus luteum (Brannstrom and Norman 1993; Brannstrom et al 1994a; Best et al 1996) and products of luteal macrophages may have angiogenic properties (Brannstrom and Norman 1993). TGF-β (transforming growth factor beta), TNF-α (tumour necrosis factor alpha), interleukin 1 and interleukin 6 are present in the corpus luteum and may be indirectly angiogenic (Roby and Terranova 1989; Motro et al 1990; Ji et al 1991; Vassali 1992; Simon et al 1994). Although these factors promote angiogenesis in vivo (Koos 1989), in vitro studies demonstrate no effect on endothelial cell proliferation, migration or protease synthesis (Brannstrom and Norman 1993).

In tumour tissues angiogenesis is regulated by an interplay between angiogenic and antiangiogenic factors from tumour cells and the circulation (Folkman 1995; Holmgren et al 1995). No study has yet addressed whether antiangiogenic factors are present in the corpus luteum. If present, these agents may interact with angiogenic mediators to regulate vessel formation in the corpus luteum.

1.5.6 Non secreted factors

Non-secreted and structural factors may be important in the regulation of luteal angiogenesis.

The matrix of the corpus luteum plays a role in the formation of new blood vessels (Duncan et al 1996a;b;c). Degradation of the extracellular matrix is essential for angiogenesis. VEGF induces production of proteases by endothelial cells, but steroidogenic luteal cells also produce matrix metalloproteinases (Duncan et al 1996a) which break down the extra cellular matrix. The major protein product of steroidogenic luteal cells is TIMP (tissue inhibitor of metalloproteinases) (Duncan et al 1996b), which inhibits breakdown of the matrix. These high levels of TIMP may be important for the control of angiogenesis in the corpus luteum.

Blood vessel growth is dependent on proliferation of endothelial cells, but changes in the rate of death of endothelial cells will also affect angiogenic rates. Different types of cell death have been described in endothelial cells in ovine and human corpora lutea (O'Shea et al 1977; Modlich et al 1996; Fraser et al 1995). Endothelial cell death may be influenced by local production of growth hormones from steroidogenic or immune cells. Changing rates of cell death may represent another regulatory point in the control of the luteal vasculature.

In addition to the extent of the luteal vasculature, the blood flow to the corpus luteum may also be regulated by vascular tone. Vasoconstriction may result in reduced luteal flow during luteal regression, and uterine PGF2α may reduce luteal flow by this mechanism in domestic species. In primates, local factors such as nitric oxide production, hypoxia and prostaglandins may bring about changes in vascular tone and luteal flow.

1.5.7 Antiangiogenic agents

Antiangiogenic agents are being evaluated for use as cancer treatments (Toi 1995). Administration of these agents in a mouse model of lung carcinoma causes a significant reduction in tumour size. Tumour shrinkage is not mediated by a decrease in proliferation, but by a significant increase in the rate of cell death in the tumour (Holmgren et al 1995). This may be directly due to a decreased blood supply, or to more indirect factors such as decreased local production of growth factors.

In a similar way changing rates of angiogenesis in the corpus luteum may be associated with changing rates of cell death during luteal regression and maintenance.

1.6 Cell death in the corpus luteum

1.6.1 Luteal growth and cell death

A high rate of cellular proliferation, particularly in endothelial cells, is characteristic of the corpus luteum throughout its functional lifespan. Proliferation in the gland is tightly regulated and the corpus luteum is rarely the site of malignant transformation. Cell death plays an opposite but complementary role to mitosis in regulation of cell populations in other tissues (Wyllie 1992). Cell death may balance proliferation in the corpus luteum and changes in the rate of cell death in endothelial and steroidogenic cells may allow growth in early pregnancy or regression at the end of the cycle.

1.6.2 Cell death in the corpus luteum

Early descriptions of luteal morphology include features that today are recognised as characteristic of cell death. Two main morphological forms of cell death have been identified in the corpus luteum; (1) apoptosis and (2) necrosis (O' Shea et al 1977; Sawyer et al 1990; Fraser et al 1995)

Necrosis involves varying numbers of adjacent cells and usually follows a pathological insult to the tissue, while apoptosis is concerned with the deletion of single cells in the midst of healthy tissue (Kerr et al 1972; Ueda and Shah 1994). Apoptosis is observed as a normal part of developmental and physiological processes (Kerr et al 1972; Ueda and Shah 1994), and is also involved in regression of malignant tumours (Wyllie 1992; Holmgren et al 1995; Thompson 1995).

Increasing morphological and biochemical evidence suggests that apoptosis may be important in control of luteal structure and function.

1.6.3 Apoptosis- historical perspective

The first morphological features of selective cell death in any tissue were described in the ovary by William Flemming who in 1885 noted that the epithelial lining of regressing follicles contained cells with fragmenting nuclei. Flemming called this process chromatolysis, referring to the fragmented

nucleus which usually disappeared. A further description of the same morphological changes in lactating mammary gland was published the following year (Nissen 1886). A paper published in 1914 by Graper stated that "chromatolysis must exist in all organs in which cells must be eliminated" implying that this type of cell death was physiological. The concept of cell suicide was first proposed in the 1950s when DeDuve speculated that cells might be killed from within (quoted in Manjo and Joris 1995). The term apoptosis was first used to describe these changes in 1972. In ancient Greek the word apoptosis describes leaves falling one by one from trees in autumn (Kerr et al 1972).

1.6.4 Apoptosis and programmed cell death

The phenomenon known as programmed cell death refers to situations where cells are programmed to die at a fixed time (Bowen 1993; Manjo and Joris 1995). Such cells usually but not always die by apoptosis, for example apoptosis is responsible for the death of cells between digits in the embryonic hand and foot (Saunders and Fallon 1967), but is not observed during the massive programmed cell death observed during formation of the nervous system (Server and Mobley 1991). Similarly, extrinsic factors such as ionising radiation can induce apoptosis in cells where it has not been pre-programmed (Lowe et al 1993a;b). Programmed cell death refers to the set of commands within a cell that will signal it is time for that cell to die while apoptosis refers to the type of death that cell then undergoes.

General characteristics of apoptosis are well established and include morphological and biochemical characteristics. Cells undergoing apoptosis shrink and become denser. The chromatin becomes pyknotic and is packed into smooth masses applied against the nuclear membrane creating semi-circular shapes. The nucleus of the cell may then break up. The cell emits processes which may break off and form apoptotic bodies, which are

phagocytosed by tissue macrophages and neighbouring cells (Kerr et al 1972; Wyllie et al 1980; Manjo and Joris 1995). In contrast to necrosis, there is little or no swelling of intracellular organelles, and apoptotic cells do not attract lymphocytes and macrophages. However when apoptosis occurs on a large scale as seen in the embryo, an influx of phagocytes is involved with disposal of apoptotic bodies (Duvall et al 1985; Savill et al 1990). The process of apoptosis, from the appearance of the first morphological changes to the phagocytosis of cellular remnants, is extremely rapid, with a duration of between 15 minutes and 1 hour (Manjo and Joris 1995). This may explain scanty morphological evidence of apoptosis in some tissues where this form of cell death is thought to occur at high rates.

Biochemically, apoptosis is associated with a characteristic pattern of DNA degradation. DNA is wrapped around protein cores known as histones, with one histone holding 180-200 base pairs of DNA (Bortner et al 1995). During apoptosis, the DNA is cleaved between histones, so the segments of DNA remaining are multiples of 180-200 base pairs long. Resolution on a gel gives rise to the typical laddering pattern associated with apoptotic cell death (Wyllie 1980). In apoptotic cells DNA is thought to be degraded by an endogenous calcium and magnesium dependant endonuclease which bears many similarities to DNAase 1 (Peitsch et al 1994).

This contrasts with the pattern of DNA degradation associated with necrosis where DNA can be cleaved at any point. When resolved on a gel DNA from necrotic tissue results in a smear of different sized DNA fragments (Wyllie 1993).

DNA degradation in apoptosing cells may also be examined by 3' end labelling where a biochemical tag is attached to broken ends of degraded DNA (Gavrieli et al 1992; Ansari et al 1993). This method has been used to identify apoptotic cells in tissue sections. However this method is not specific for apoptosis as DNA fragmentation also occurs at a late stage during necrotic cell death .

1.6.5 Apoptosis in reproductive tissue

Apoptosis has been observed in reproductive tissues and may be regulated by hormonal factors.

In the endometrium apoptosis is observed in the glandular epithelium and increases in frequency as the secretory phase of the menstrual cycle progresses, peaking during menstruation (Tabidzadeh et al 1994; Gu et al 1994). Progesterone and TNFα may be involved in cyclic regulation of endometrial apoptosis (Koh et al 1995). There is also evidence that apoptosis is involved in decidual regression during blastocyst implantation (Ackali et al 1996).

The first historical description of morphological features of apoptosis was concerned with ovarian follicles (Flemming 1885). Apoptosis is now thought to be responsible for perinatal germ cell loss (Coucouvanis et al 1993; Pesce and de Felicici 1994) and follicular atresia (Tilly 1993; Ratts et al 1995; Tilly and Ratts 1996). Gonadotrophins are effective inhibitors of apoptosis occurring in granulosa cells of follicles deprived of trophic support in vitro (Chun et al 1994; Tilly and Tilly 1995). Progesterone has also been implicated in the control of follicular apoptosis (Luciano et al 1994).

Other examples of hormonally regulated apoptosis in the reproductive system include the inhibition of apoptosis by testosterone in the prostate gland (Kyprianou et al 1990) and by oestrogen in the breast (Wang and Phang 1995).

Apoptosis occurs in a cyclical manner in the female reproductive system and can be influenced by hormonal factors. Apoptosis may be important in regulating luteal lifespan. Endocrine, paracrine or autocrine factors may play a role in the control of apoptosis in the corpus luteum.

1.6.6 Apoptosis in the corpus luteum

There is growing evidence to support the hypothesis that apoptosis may occur during luteal regression. The rate of cell death in the gland may be fundamental for the control of its lifespan, and the constant length of the luteal phase suggests that death may be pre-programmed into these cells.

Morphological features consistent with apoptosis have been observed in corpora lutea of domestic species, marmoset monkeys and women (Corner 1956; Sawyer et al 1990; Young et al 1996). DNA cleavage characteristic of apoptosis was first described in luteinised rat granulosa cells in vitro (Zeleznik et al 1989) and was followed by the demonstration of oligonucleosome formation in regressing cow corpora lutea (Jeungel et al 1993; Rueda et al 1995). Subsequent investigations have shown that DNA laddering also occurs in regressing corpora lutea from other domestic species, marmoset monkeys and women (Shikone et al 1996; Young et al 1996).

Luteal apoptosis has also been examined by 3'end labelling studies. This method has revealed that positive cells in cow, sheep, monkey and human are more numerous during luteal regression (Zheng et al 1994; Rueda et al 1995; Funayama et al 1996; Shikone et al 1996; Young et al 1996). Studies in women have observed a low incidence of 3' end labelled cells in early pregnancy suggesting that down regulation of apoptosis may be important for maintaining the structure and function of the gland in early pregnancy. eCG has been shown to reduce apoptotic death in cultured granulosa cells (Tilly et al 1995), and it is possible that hCG could also lower apoptotic rates in the corpus luteum in women.

The majority of apoptotic cells in the corpus luteum, as assessed by morphology and 3'end labelling, are of steroidogenic origin. Apoptotic endothelial cells have also been described in morphological studies (O'Shea et al 1977; Fraser et al 1995). Changing rates of apoptosis in both of these cell compartments may be implicated in luteal regression and maintenance.

1.6.7 Regulation of apoptosis by internal factors

The role of hormones in regulating apoptosis has already been discussed, but apoptosis may also influenced by local factors such as growth factor production (Lynch et al 1986; Araki et al 1990; Duke and Cohen 1990; Williams et al 1990) and trophic stimulation from neighbouring cells (Raff 1992). The fate of the cell may also be controlled by internal factors such as proto-oncogene expression. Many proto-oncogenes which have varying effects on the fate of a cell have been identified. The precise mechanisms by which these proto-oncogenes influence the fate of the cell are unknown, but they have well conserved actions throughout phylogeny of species (Wyllie 1992; Hengartner and Horvitz 1994). Although cell death may be induced solely by proto-oncogene expression external factors may interact with proto-oncogenes to induce apoptosis. A high turnover state may be induced in a cell by expression of proto-oncogenes such as c-myc (Evan et al 1992; Wyllie 1992; 1993; Harrington et al 1994). In this situation an excess of local growth factors may lead to proliferation, where absence of growth factors may lead to apoptosis (Wyllie 1992; Fandini et al 1992). In the corpus luteum, this implies that apoptosis may be regulated by proto-oncogene expression within steroidogenic and endothelial cells but also by hormone and growth factor availability.

1.6.8 Bcl-2 and apoptosis

The bcl-2 proto-oncogene (B cell lymphoma/leukaemia -2) was identified at the breakpoint site of the t(14;18) translocation that is associated with follicular lymphoma and its expression prevents cell death induced by a wide range of apoptotic stimuli (Tsujimoto and Croce 1986). Immunocytochemical localisation of bcl-2 within cells has revealed its association with mitochondrial membranes, endoplasmic reticulum and the nuclear envelope (Hockenbery et al 1990; Monaghan et al 1992; Riparbelli et al 1995). The precise mechanism of action of bcl-2 is not known but it can function in cells which lack a nucleus and also in

those which lack mitochondrial DNA (Jacobson et al 1993;1994). It has been proposed that bcl-2 may act as an antioxidant or free radical scavenger to protect cells from the damaging effects of reactive oxygen species (Kane et al 1993; Hockenbery et al 1993). Bcl-2 expression has been shown to be topographically restricted to tissue characterised by high rates of programmed cell death (Hockenbery et al 1991).

1.6.9 Bcl-2 in reproductive tissues

Bcl-2 is particularly interesting as its expression prevents cell death induced by a wide range of apoptotic stimuli and has been implicated in the selection of ovarian follicles and regulation of apoptosis in the endometrium (Gompel et al 1994; Tilly et al 1995; Tilly 1996).

Bcl-2 is expressed in glandular epithelium and spiral arteries in the endometrium and its expression is maximal at the end of the proliferative phase (Otsuki et al 1994). With declining bcl-2 levels in the secretory phase apoptosis appears in the endometrial glands. The cyclicity of bcl-2 expression suggests that it may be regulated by hormonal factors. This is supported by the observation that variations in expression of bcl-2 occur after treatment with oestrogen and progesterone in endometrium and breast. However the promoter region sequence of bcl-2 contains no recognised steroid regulatory elements (Akcali et al 1996).

Bcl-2 is expressed at lower levels in decidua of women with failing pregnancies when compared to those with normal pregnancies, and may be involved in survival of syncytiotrophoblast during placentation (Lea et al 1997). Bcl-2 expression has been examined in rat follicles. Bcl-2 is expressed in granulosa cells but levels of expression do not change during selection for dominance or atresia (Tilly et al 1995).

Ablation of functional bcl-2 through targeted disruption of the gene in mice (gene knockout) leads to significantly fewer oocytes and primordial follicles in

the postnatal ovary, but granulosa cell apoptosis is not affected (Ratts et al 1995).

1.6.10 Bcl-2 and bax

In recent years it has become apparent that bcl-2 is one member of a family of structurally similar proto-oncogenes which act to promote or suppress apoptosis (Nunez and Clarke 1994). The homology among the bcl-2 related proteins is concentrated in two regions, termed BH1 and BH2 (Yin et al 1994). In addition, proteins of the bcl-2 family contain a stretch of hydrophobic amino acids at their C terminal which may be important for membrane insertion (Nguyen et al 1993). At the outset of the work described in this thesis, only bcl-2 and bax had been described.

Bax protein was identified by immunoprecipitation of bcl-2. Bax is a 21kDa protein with 21% homology to bcl-2. Bax forms heterodimers with bcl-2, and induces apoptosis (Oltvai et al 1993). It is unclear which of bcl-2 or bax is the effector protein or which is the blocking protein. It appears that the rate of apoptosis in a tissue is more closely related to the bcl-2 /bax ratio than to absolute levels of either of the proto-oncogenes. Bax is distributed in a wider range of tissues than bcl-2 (Krajewski et al 1994a), implying that it may interact with other bcl-2 family members or that bax may have alternative functions not involved with apoptosis. Interactions between bax and bcl-2 are thought to be important in ovarian follicle selection. Although bcl-2 levels remain constant throughout follicular development, bax levels are higher in follicles destined for atresia than those which become dominant (Tilly et al 1995). In other tissues such as endometrium bax levels remain unchanged while bcl-2 levels decrease to induce apoptosis. (McLaren et al 1997) The bcl-2/ bax ratio, rather than the absolute level of either is the most important indicator of the fate of a cell.

The pattern and distribution of bcl-2 and bax expression throughout the lifespan of the corpus luteum and in early pregnancy are not known for any species.

1.6.11 Overview

Apoptosis may be a controller of luteal growth and lifespan. Programmed cell death is controlled by the expression of a number of proto-oncogenes. Patterns of proto-oncogene expression in the corpus luteum have not been characterised. Endocrine, paracrine and autocrine factors may influence the expression of apoptotic proto-oncogenes within the corpus luteum.

1.7 The immune system and luteal function

1.7.1 Immune cell interactions in the corpus luteum

Immune cells are present in the corpus luteum and have previously been implicated in structural luteal regression. However these cells may be important not only during regression, but throughout the lifespan of the gland.

A role is emerging for immune cells in the control of ovulation (Brannstrom and Norman 1993) and this may extend to a regulatory role in the corpus luteum. Suppression of the immune system can disrupt ovarian function and may alter luteal lifespan (Wang et al 1993; Alila and Hansel 1984)

Interactions between cells of the immune and reproductive systems may influence steroidogenic output (Halme et al 1985; Emi et al 1991; Yan et al 1993) and blood vessel growth (Koch et al 1992; Vassali et al 1992), and may regulate the balance between cell death and proliferation in the corpus luteum (Fukuoka et al 1989; Wang et al 1991; Redmer et al 1996). It is possible that changes in immune cell number, distribution or cytokine production may influence the function of the corpus luteum during luteal regression and maintenance. Leukocytes may also be important for formation and continuing function of the mature corpus luteum.

1.7.2 Immune regulation of luteal function.

Cells of the immune system may regulate luteal function. Suppression of immune function using glucocorticoids in the rat prolongs luteal function in hysterectomised animals, although immunosuppression does not counter PGF2α induced luteal regression (Wang et al 1992). The effects of immunosuppression on luteal function in women are unknown.

Conversely, in vitro studies have demonstrated that peripheral blood leukocytes or their secretory products can increase progesterone production from luteinised human granulosa cells (Emi et al 1991). Hence the immune system may be capable of both stimulating and inhibiting luteal function under different circumstances.

Macrophages (Gillim at al 1969; Wang et al 1992), neutrophils (Brannstrom et al 1994a), and lymphocytes (Best et al 1996) have been identified in human corpora lutea and studies indicate that numbers of macrophages increase in spontaneous luteolysis in the human (Lei et al 1991; Brannstrom et al 1994a) while numbers of neutrophils increase during PGF2α mediated luteal regression in the rat (Brannstrom et al 1993; 1994b). Free radical production by neutrophils may have a luteolytic effect as neutrophils incubated with luteal cells from rat decreased LH stimulated progesterone production but this effect was blocked by SOD and catalase(Peperell et al 1992). In addition, production of intracellular ROS by cultured luteal cells increased in the presence of activated neutrophils. No increase in neutrophil number is observed during spontaneous luteolysis in women (Brannstrom et al 1994a).

Recent studies by Duncan and colleagues (Duncan et al 1998) have examined macrophage distribution during luteal maintenance and regression in women. Macrophages were numerous in the late luteal phase and at this time the majority of macrophages were situated at the junction of the theca and granulosa lutein cells. Macrophage numbers fell significantly following hCG treatment. It is not known whether other leukocyte subsets vary with changing luteal function.

1.7.3 Secretory products of leukocytes

Immune cells can affect luteal function in a number of ways. As well as their role in cell death and phagocytosis, cells of the immune system may break down the extracellular matrix of the gland or affect steroidogenesis by free radical production (Behrman et al 1993).

Cytokines are an important group of factors secreted by leukocytes which can cause changes in the surrounding cells. These factors may act directly on granulosa and theca lutein cells to influence steroidogenesis (Sjorgen et al 1991; Fairchild et al 1992; Fukouka et al 1992), or act indirectly to affect the luteal vasculature, act as growth factors or alter rates of programmed cell death in the corpus luteum (Yan et al 1993; Tilly 1996).

Tumour necrosis factor alpha (TNFα) is secreted by macrophages and T lymphocytes and has been identified in corpora lutea of domestic animals and humans (Roby et al 1990). In vitro experiments in the cow suggest that this cytokine can suppress progesterone biosynthesis (Fairchild et al 1992). Progesterone biosynthesis by hCG stimulated luteinised granulosa cells is also decreased in women in vitro by IL-2, which is secreted by T cells (Wang et al 1991). In contrast IL-1, secreted by neutrophils and macrophages has been shown to increase basal and hCG stimulated progesterone synthesis in luteinised human granulosa cells (Sjorgen et al 1991).

1.7.4 Steroidogenic cells may produce chemotactic factors

Macrophages are most prevalent during luteal regression and are less abundant in the human corpus luteum after hCG stimulation (Duncan et al 1998). As luteal macrophages do not express the LH receptor (Duncan et al 1996d), the change in macrophage numbers associated with hCG treatment may be mediated by a steroidogenic cell product.

MCP-1 (monocyte chemoattractant protein-1) has been localised to steroidogenic luteal cells in the rat (Hosang et al 1994). Rising levels of this peptide are associated with a macrophage influx during functional luteolysis in the rat (Townson et al 1995;1996). MCP-1 production is inhibited by physiological levels of progesterone (Kelly et al 1997) and it is possible that the decline in progesterone associated with functional luteal regression allows upregulation of MCP-1 production. This may in turn trigger an influx of macrophages necessary for structural regression of the corpus luteum. Patterns of MCP-1 production in the human corpus luteum are unknown.

Similarly, IL-8 is chemotactic and activating for neutrophils in vivo and in vitro (Baggiolini et al 1989) and is also angiogenic (Koch et al 1992). IL-8 expression has been identified in cultured human granulosa lutein cells (Arici et al 1996). The pattern of IL-8 expression with changing luteal function has not been investigated, but steroidogenic cell IL-8 secretion may allow neutrophils to enter the gland at times where proteolytic digestion is required for tissue remodelling.

1.7.5 Overview

The cells and secreted factors of immune, steroidogenic and endothelial compartments interact in complex ways in the corpus luteum. Immune cells are present throughout the luteal phase and numbers may rise in luteal regression. This increase may be mediated by chemotactic factors from steroidogenic cells. With the potential to affect the function and growth of endothelial and steroidogenic cells the immune system may be an important regulator of luteal function throughout the lifespan of the gland.

1.8 Summary

Luteal cells may interact directly or by means of secreted products. Such interactions may change the rate of cell growth or death in the gland, and may alter its steroidogenic potential, its blood supply or its cellular composition.

Post LH receptor mechanisms regulating luteal function and lifespan are likely to involve complex interactions between the different cell types of the gland. This thesis sets out to dissect physiological processes which may be involved in luteal regression and maintenance in women, and examines how the different cell types of the gland may influence these processes.

CHAPTER 2

TISSUE COLLECTION AND CHARACTERISATION

2.1 Introduction

Studies on cellular interactions in the human corpus luteum were undertaken on human tissue. Ovarian biopsies were obtained from premenopausal women undergoing hysterectomy for benign conditions. To simulate early pregnancy, and allow comparisons between luteal maintenance and regression hCG (Profasi, Serono, Randolph, MA, USA) was administered to maintain the lifespan of the corpus luteum. Ethical approval for hCG treatment and removal of luteal tissue was sought and obtained from the reproductive subdivision of Lothian regional ethics committee.

2.2 Recruitment of donors: Controls.

Corpora lutea were obtained from premenopausal women who were expected from dates of their last menstrual period to be in the luteal phase of the menstrual cycle at the time of their planned hysterectomy.

2.2.1 Inclusion and exclusion criteria

Inclusion criteria for the study were as follows:

a) Age 18-45 years

b) Awaiting elective abdominal hysterectomy.

c) Regular menstrual cycles.

Exclusion criteria were as follows:

a) Suspicion of malignant disease

b) Hormonal medication

c) Concurrent disease

d) History of infertility

2.2.2. Recruitment protocol

Suitable patients were identified from gynaecology waiting list clinical notes. A patient information letter (figure 2.1) was sent to all patients identified as potentially suitable. This letter briefly outlined the purpose of our studies and gave a telephone number which interested women could contact. On contacting our group requirements of the study were explained in detail and suitability to participate was determined. In particular, the date of the last menstrual period was ascertained. If the date of planned surgery was due to fall in the luteal phase of the cycle, daily urine collection was commenced for serial LH tracking allowing the day of the LH surge to be determined. A detailed medical history was obtained and the subject signed a standard consent form. Venous blood was sampled for estimation of serum progesterone on the day of surgery.

2.3 Recruitment of donors: hCG treatment

In addition to the control group, a group of 14 women who were scheduled to have surgery during functional luteal regression received hCG treatment in order to simulate the endocrine effects of early pregnancy.

2.3.1 Treatment protocol

Patients were initially recruited by letter as detailed for control subjects. The patient information sheet for the treatment group is shown in figure 2.2. In 8 subjects who were scheduled for surgery 24 and 30 days after their last menstrual period, urine samples collected daily for LH tracking were analysed between 2 and 5 days after the expected day of ovulation. The day of the urinary LH peak was taken as day 0, with subsequent serum progesterone estimations confirming ovulation. Treatment with hCG (Profasi, Serono) to mimic the endocrine effects of early pregnancy was commenced on day 7 of

the luteal phase. The hCG was administered by intramuscular injection for between 5 and 8 consecutive days in incremental doses beginning with 125IU per day then doubling daily to a maximum of 16,000IU. Administration of hCG over more than 8 days was precluded by pain associated with the injection of increasing volumes of hormone. Venous blood was collected on alternate days during hCG administration as well on the day of surgery and plasma was stored at -20^{o}C for subsequent radioimmunoassay for progesterone. The serum concentrations of hCG, progesterone and inhibin produced by this regime have been shown previously to be similar to those seen in normal pregnancy (Illingworth et al 1990; Illingworth et al 1996). hCG treatment lasted for a mean of 7.5 days (range 5-8 days). Serum progesterone levels rose significantly ($p<0.05$) from a mean of 34.5 nmol/l prior to treatment to a mean of 43.9 nmol/l on the day of operation, confirming luteal rescue in each case (figure 2.3).

2.3.2 Inclusion and exclusion criteria

Inclusion criteria for hCG treatment were as follows:-

a) Age 18-45 years

b) Awaiting elective abdominal hysterectomy

c) Regular menstrual cycles

d) Due to have surgery 24-30 days after last menstrual period

Exclusion criteria for hCG treatment were as follows:-

a) Suspicion of malignant disease

b) Hormonal medication

c) Concurrent disease

d) History of infertility

2.4 Characterisation of luteal age and function

In order to correlate changes in the corpus luteum with the functional status of the gland the time (in days) since ovulation was determined for each luteal biopsy.

2.4.1 Menstrual history

A detailed menstrual history was taken prior to tissue retrieval. Subjects had regular menstrual cycles, allowing the age of the corpus luteum to be estimated.

2.4.2 Urinary LH tracking

All subjects collected daily urine samples in the cycle leading up to their operation. Approximately 5mL of urine per day was collected and stored in a domestic freezer until collection on admission to hospital. Serial urine samples were subjected to radioimmunoassay for LH by Mrs F Pitt of the MRC Reproductive Biology Unit. Urinary LH levels were expressed as LH/creatinine ratios to allow for changes in urine concentration. In all cases where a corpus luteum was obtained an LH surge was easily identified, allowing accurate luteal dating (Djahanbakhch et al 1981).

2.43. Serum progesterone determination

Venous blood was taken on the day of surgery from control group subjects to allow assessment of the functional state of the corpus luteum. Venous blood samples were collected on alternate days during hCG administration as well as on the day of surgery. Plasma was stored at -20^{o}C for subsequent radioimmunoassay for progesterone by Mrs F Pitt, MRC Reproductive Biology Unit.

2.4.4 Endometrial morphology

Endometrial samples retrieved from luteal donors at hysterectomy were fixed in 4% paraformaldehyde for 24 hours, processed and mounted in paraffin blocks. Haematoxylin and eosin stained 5μm sections cut from these blocks were dated according to the criteria of Li et al (1988) by Dr WC Duncan, MRC Reproductive Biology Unit. This gave further validation of luteal age at retrieval.

2.4.5 Assignment to early, mid, late or rescued group.

After the date of ovulation and functional state of the corpora lutea had been determined, control subjects were grouped according to luteal age as follows: early luteal, 1-4 days since ovulation (n=11); mid luteal, 5-9 days since ovulation (n=11); late luteal, 10-14 days since ovulation (n=15).
hCG treated subjects (n=14) were identified as rescued corpora lutea.

2.5 Collection and storage of tissue

2.5.1 Removal at laparotomy

At laparotomy the corpus luteum was identified and enucleated from the ovary. This was characteristically accompanied by only minimal blood loss. One or two stitches were required to repair the defect in the ovary in approximately 10% of cases. In cases where oopherectomy was planned, the corpus luteum was enucleated from the ovary prior to ligation of the ovarian vessels. Immediately after removal the corpus luteum was cut into radial sections which were processed for either fixing or freezing.
After removal of the uterus at laparotomy a longitudinal incision was made with a scalpel from the fundus to the cervix until the uterine cavity was exposed. Endometrial samples were retrieved from the uterine cavity with forceps and fixed in 4% paraformaldehyde for subsequent dating studies.

2.5.2 Frozen tissue

Radial sections of corpus luteum were frozen using two different methods. Sections were placed in labelled cryotubes and frozen immediately in liquid nitrogen for subsequent storage at -70°C. A further section of corpus luteum was coated in OCT tissue medium, frozen on a slurry of dry ice and isopentane and subsequently stored at -70°C for frozen section use.

2.5.3 Fixed tissue

Radial sections of corpus luteum and endometrial biopsies were fixed for 24 hours in 4% paraformaldehyde in 0.1M phosphate buffered saline pH7.4. After tissue processing, sections were embedded in paraffin blocks and stored at room temperature for subsequent use.

2.6 Analysis of tissue obtained

2.6.1 Number of donors per year

Numbers of luteal biopsies obtained declined from 28 in the first year of the study (1993) to only 3 in the last full year of the study (1996) (figure 2.3). A number of factors accounted for this fall. Firstly, the number of patients identified from clinical notes as suitable for luteal donation fell from 206 in 1993 to 100 in 1996. The proportion of replies to recruitment letters did not vary significantly over the course of the study.

Subjects who replied to the recruitment letter but were found to be unsuitable after detailed discussion and urinary LH tracking rose from 39% in 1993 to 70% in 1996. Subjects who were expected to be in the follicular phase of their cycle on the day of surgery accounted for the majority of these cases (65%). Other reasons for unsuitability included hormonal treatment which had not been previously identified from clinical notes (22%), laparoscopically assisted vaginal hysterectomy (8%) and high risk status (2%). This may reflect increasing use of

hormonal treatments for menstrual symptoms or an increasing trend towards laparoscopically assisted vaginal hysterectomies (LAVH). LAVH is a procedure which is unsuitable for luteal retrieval as the corpus luteum will have been avascular for approximately 45 minutes before delivery of the pelvic organs through the vagina.

The proportion of suitable subjects who had an identifiable corpus luteum at laparotomy did not vary significantly over the study period (range 39-74%).

2.6.2 Demographic details

Demographic details for luteal donors were as follows:

a) Mean age of donors was 40 years (range 29-42 years). Mean age did not change significantly over the years of the study. Mean donor age was 40 years for each of the early, mid, late and rescued groups.

This study includes a large proportion of women who are of older reproductive age (median age 40 years). However all luteal donors demonstrated midcycle urinary LH peaks and all subjects had ovulatory progesterone concentrations apart from the late luteal group where the low concentration would be anticipated. In addition, Klein et al, 1996 has shown that while older women have marked differences in early phase FSH and inhibin B concentrations compared with younger women, there is no difference in the function of either the dominant follicle or the corpus luteum.

b)The commonest indication for hysterectomy in luteal donors was menorrhagia (54%), followed by fibroids (22%) and endometriosis (14%). Other indications for hysterectomy are shown in figure 2.4.

c) The majority of luteal donors (73%) underwent a total abdominal hysterectomy with ovarian conservation. Total abdominal hysterectomy and bilateral salpingo-oophorectomy was performed in 17% of donors, while 7% of donors underwent total hysterectomy with a unilateral oophorectomy. Subtotal hysterectomy with ovarian conservation was performed in 2% of donors. A

further 2% underwent subtotal hysterectomy with bilateral salpingo-oophorectomy.

2.6.3 Assessment of luteal function.

Venous blood was obtained for serum progesterone determination on the day of operation for the control group, and every 48 hours as well as on the day of operation during hCG treatment. Mean serum progesterone levels rose from 21.8ng/mL (confidence limits 16.4 - 27.2nmol/L) in the early luteal phase to 34.5ng/mL (confidence limits 26.8 - 42.2nmol/L) in the mid luteal phase. Levels fell significantly to 18.7nmol/L (confidence limits 14.1 - 23.3nmol/L) in the late luteal phase, $p<0.05$). hCG treatment was associated with significant increase in serum progesterone to a mean of 43.9nmol/L (confidence limits 39.0- 48.8 nmol/L, $p<0.05$) in the rescued group (figure 2.5).

Figure 2.1

Patient information sheet (control group)

THE MECHANISM OF PREGNANCY RECOGNITION IN WOMEN

PATIENT INFORMATION SHEET

CONTROL GROUP

Research into the establishment of early pregnancy in women.

What is the research about?

The establishment of early pregnancy is a very important process in women and problems such as infertility and early miscarriage are unfortunately very common. It is known that a woman's body will recognise pregnancy as a result of a hormone called hCG which is produced by the early embryo. This hormone hCG stimulates the ovary leading to all the other changes of pregnancy. However, we still do not understand the critical processes by which the ovary recognises this hCG and thus acts to maintain the pregnancy. This research is therefore intended to improve our understanding of early pregnancy.

What will be involved?

Each month at the time of a period, the ovary forms new cells which produce hormone and then release an egg at the time of ovulation. If pregnancy does not occur, these cells continue to produce hormone for 14 days before they die at the time of the next period. In this research we propose to remove these cells (which will die anyway) from the ovary at the time of your operation. This will extend the operation by approximately five minutes but will not add anything to the discomfort normally experienced at such an operation. In addition there will be no effect on your future hormone production.

In addition you will be asked to collect urine samples during the days leading up to your operation in order that we can measure your normal hormone production.

Your participation or otherwise in this research will not in anyway affect the treatment you will receive at the Royal Infirmary and you will be free to withdraw at any time.

Further information is available from Dr Faye Rodger, Clinical Research Fellow, MRC Reproductive Biology Unit.

Figure 2.2

Patient information sheet (hCG treatment group)

THE MECHANISM OF PREGNANCY RECOGNITION IN WOMEN

PATIENT INFORMATION SHEET

hCG TREATMENT GROUP

Research into the establishment of early pregnancy in women.

What is the research about?

The establishment of early pregnancy is a very important process in women and problems such as infertility and early miscarriage are unfortunately very common. It is known that a woman's body will recognise pregnancy as a result of a hormone called hCG which is produced by the early embryo. This hormone hCG stimulates the ovary leading to all the other changes of pregnancy. However, we still do not understand the critical processes by which the ovary recognises this hCG and thus acts to maintain the pregnancy. This research is therefore intended to improve our understanding of early pregnancy.

What will be involved?

Each month at the time of a period, the ovary forms new cells which produce hormone and then release an egg at the time of ovulation. If pregnancy does not occur, these cells continue to produce hormone for 14 days before they die at the time of the next period. In this research we propose to remove these cells (which will die anyway) from the ovary at the time of your operation. This will extend the operation by approximately five minutes but will not add anything to the discomfort normally experienced at such an operation. In addition there will be no effect on your future hormone production.

In order to investigate how the ovary responds to hCG, you will be given small amounts of hCG by daily injection for up to eight days. This is a naturally occurring female hormone which is in regular use in infertility treatment at much larger doses with no significant side-effects. In particular, there will be no

pregnancy symptoms at this very low dosage and no long term effects on your body.

Finally, blood samples will be required on alternate days in the days leading up to your operation and you will be asked to collect daily urine samples in order that we can measure your normal hormone production.

Your participation or otherwise in this research is entirely voluntary and will not in any way affect the treatment that you will receive at the Royal Infirmary and you will be free to withdraw at any time. We will not be able to make any payments to volunteers for helping us with this research but will meet the cost of any expenses incurred by volunteers.

Figure 2.3
Luteal biopsies obtained over course of study

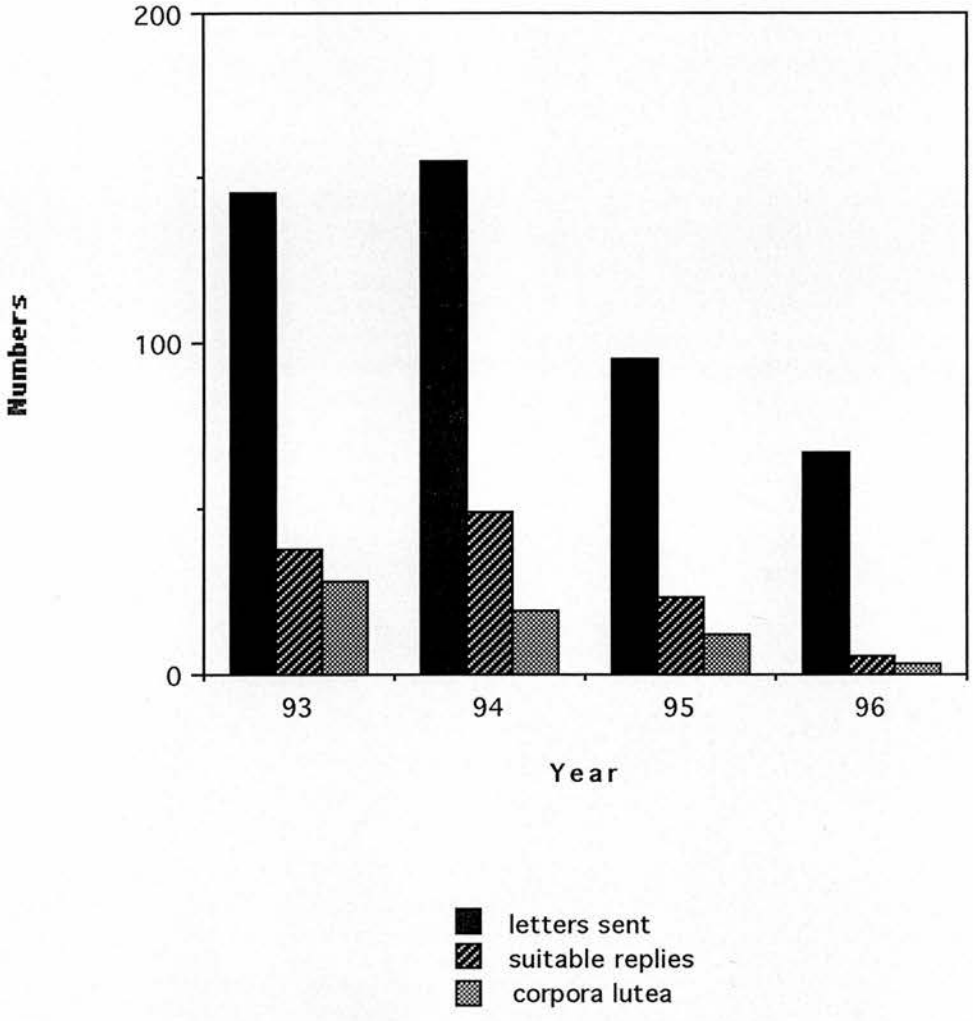


Figure 2.4
Indications for hysterectomy in CL donors

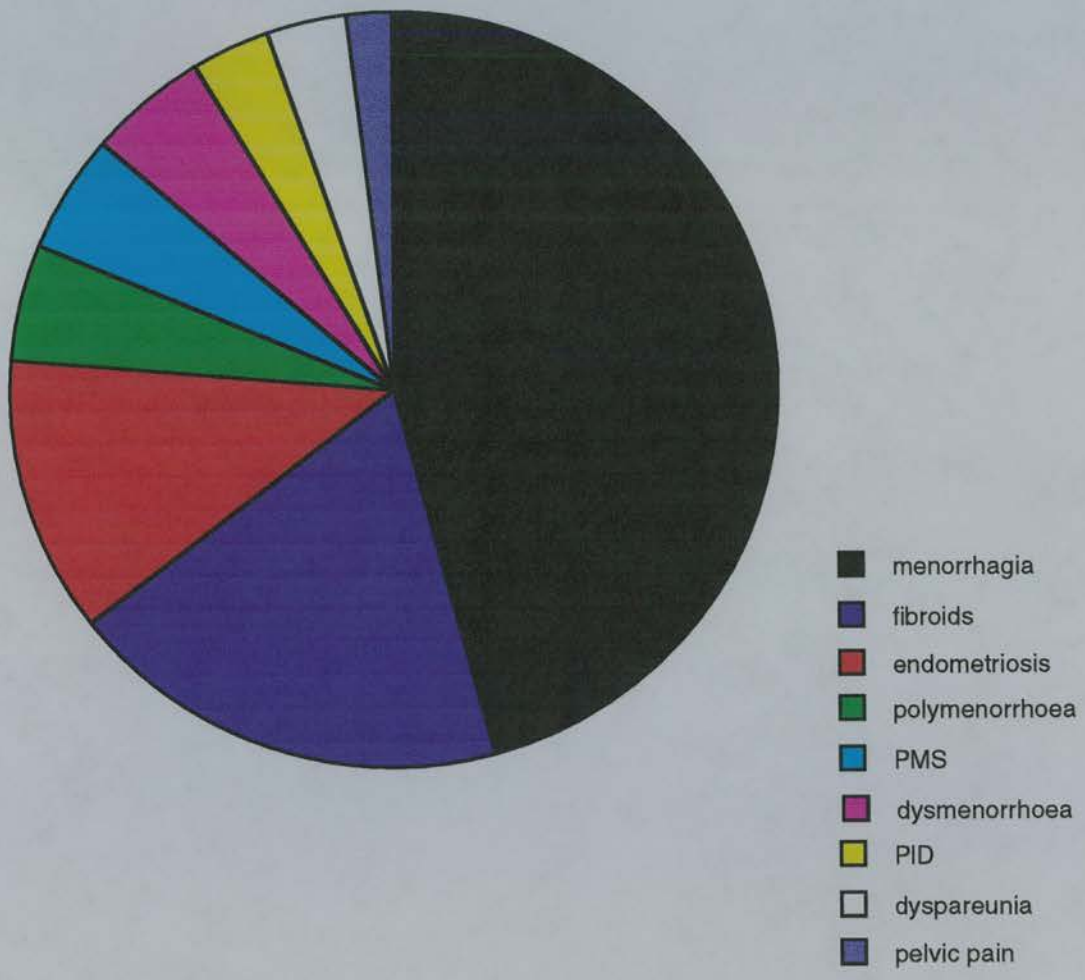
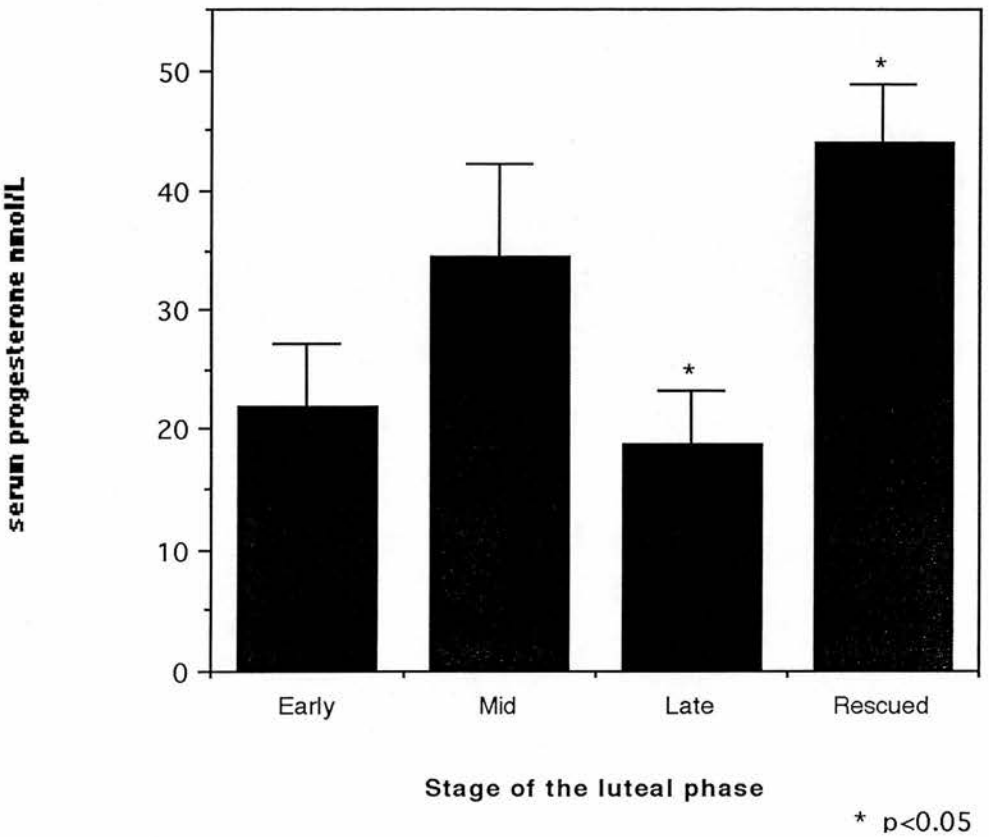


Figure 2.5
Serum progesterone throughout the luteal phase



* $p<0.05$

CHAPTER 3

ENDOTHELIAL CELL PROLIFERATION IN THE HUMAN CORPUS LUTEUM

3.1 Introduction

3.1.1 Luteal vasculature

The contribution of local vasculature to regulation of luteal function in women is unclear but evidence from other species suggests that control of endothelial cell function may be important. The corpus luteum has the highest blood supply per unit weight of any organ in the body (Bruce and Moor 1976; Wiltbank et al 1988). Approximately 50% of cells in the mature rat corpus luteum are endothelial cells resulting in the majority of steroidogenic cells being in contact with at least one capillary (Dharmarajan et al 1985; Reynolds et al 1992; Zheng et al 1993). Luteolysis in the sheep is associated with a rapid diminution in the luteal blood flow which falls at a much greater rate than tissue weight implying that luteal regression is preceded by changes in the vasculature (Bruce and Moor 1976). Although the prostaglandins which effect ovine luteolysis exert an action on blood vessel tone, it is possible that declining numbers of blood vessels may also be involved.

3.1.2 Clinical studies

There is indirect evidence to link vascular supply with luteal function in women. Ultrasound measurements of blood flow in women have demonstrated a positive correlation between successful pregnancy and low vascular resistance thus suggesting that high luteal blood flow may be required for early pregnancy support (Baber et al 1988).

3.1.3 Angiogenesis and luteal function

Blood vessel growth may parallel the functional state of the corpus luteum. Angiogenesis has been examined in the non-pregnant human corpus luteum using immunostaining for endothelial and proliferation markers (McClure et al 1994). Although no significant changes were noted earlier in the luteal phase, a fall in endothelial cell proliferation was noted in the last five days of the cycle. Further studies examining proliferation in the human corpus luteum used P450 aromatase immunostaining as a marker of luteal function. In corpora lutea no longer expressing this enzyme, cell proliferation had fallen to a low level (Funayama et al 1996). Thus, luteal regression may be associated with declining levels of proliferation. It is not known whether endothelial cell proliferation changes during luteal rescue in early pregnancy.

3.1.4 Aims of chapter

The aim of this chapter was to investigate whether changes in function at different stages of the lifespan of the human corpus luteum are associated with either the number or the rate of proliferation of endothelial cells. In particular the effects of treatment with hCG at doses similar to those seen in early pregnancy were examined.

These questions were addressed by identifying endothelial cells using immunostaining with a monoclonal antibody directed against von Willebrand factor (an intrinsic clotting cascade protein specific for cells of endothelial lineage), and Ki67, which specifically binds to proliferating cells.

3.2 Materials and methods

3.2.1 Immunohistochemistry for von Willebrand factor

Sections (5μm) were cut onto Tespa (Sigma, Poole, UK) coated slides, air-dried at 56^{o}C, dewaxed in xylene then rehydrated in 100%, 95%, and 70%

ethanol. Sections were then incubated in 0.1% trypsin (Sigma) pH 7.4 at 37°C for 40 minutes. Endogenous peroxidase activity was inhibited using 0.3% hydrogen peroxide in methanol for 30 minutes and the sections were preabsorbed for 20 minutes with normal rabbit serum (SAPU, Carluke, UK).
In order to identify endothelial cells, a monoclonal antibody raised against human von Willebrand factor protein (vWF) was used (Dako, High Wycombe, UK). The primary antibody was applied at 1:50 dilution (0.134 mg/mL IgG concentration). For control sections, 0.134 mg/mL of mouse IgG (Vector, Peterborough, UK) was used in place of primary antibody. After incubation for 20 hours at 4°C, the secondary antibody, biotinylated rabbit anti-mouse (Vector) was applied for 45 minutes. Colouring was obtained with diaminobenzidine by means of an avidin-biotin reaction with horseradish peroxidase (Vector).

3.2.2 Immunohistochemistry for Ki67

In order to identify proliferating cells, a monoclonal antibody to the nuclear non-histone antigen, Ki67, was used on serial sections. These serial sections were prepared in a similar way to those above, but were exposed to two 10 minute cycles and one 5 minute cycle of microwave irradiation at 700w, in citrate buffer 0.01M, pH 6.0 (Shi et al 1993) in place of the trypsin digestion step. Monoclonal human Ki67 antibody (Novocastra, Peterborough, UK) was applied at a 1:50 dilution and sections were incubated at 37°C for 2 hours. Detection of the primary antibody was carried out in the same manner as for vWF. Sections of human tonsil immunostained for Ki67 in the same way as luteal tissue were included in all immunohistochemistry procedures to provide positive and negative tissue controls; in every experiment, the germinal centres of the tonsil exhibited positive immunostaining while the periphery exhibited no immunostaining.

3.2.3 Immunohistochemistry for 3β hydroxysteroid dehydrogenase

In order to determine the nature of non-endothelial cells staining positively for Ki67, dual staining for Ki67 and 3β hydroxysteroid dehydrogenase (3βHSD) was undertaken to identify the steroid-producing cells. Sections prepared as above for Ki67 immunohistochemistry were then exposed to polyclonal 3βHSD antibody raised in rabbit (gift of Prof Van Luu-The, CHUL Research Centre, Quebec) at a dilution of 1:1000 for 20 hours at 4°C. Negative control sections were exposed to normal rabbit serum at the same dilution (Dako). Antigen was detected by means of an avidin/biotin/alkaline phosphatase reaction with Vector Red (Vector).

3.2.4. Data analysis

The total number of cells exhibiting immunostaining for vWF was counted in four high power fields (x200) from opposite quadrants of the glandular component of the section. This figure was expressed as number of vWF cells per high power field. The serial sections which had been stained for Ki67 were similarly analysed. The total number of cells exhibiting immunostaining for Ki67 in the four fields were counted and expressed as number of Ki67 positive cells per high power field. In order to quantify the nature of non-endothelial cells expressing Ki67 antigen, steroidogenic cells were identified with 3βHSD antibody. The total number of cells co-localising 3βHSD and Ki67 was counted in four high power fields from opposite quadrants of the glandular component of the section and expressed as a percentage of the total number of Ki67 positive cells in that field.

In all cases the observer was unaware of the nature of the section being examined. Preliminary investigations showed that increasing the number of fields above four did not increase statistical accuracy of the estimations (For early luteal phase sections increasing number of fields examined to eight resulted in mean endothelial cell count per high power field of 120, confidence

intervals 99-141; and mean Ki67 positive cell count of 249, confidence intervals 190-308).

Differences in the number of cells expressing antigen per high power field were investigated by one-way analysis of variance using stage of the luteal phase as a between subject (corpus luteum) variable with Fischers PLSD test. The numbers of antigen-expressing cells in late and rescued corpora lutea were also compared in the same manner.

3.3 Results

3.3.1 Endothelial cells

Endothelial cells were identified by positive immunostaining for vWF in all corpora lutea examined. A similar pattern was seen in each section, with larger arterioles and venules lying in the fibrous capsule of the gland, and endothelial cells of small capillaries lying between the steroidogenic cells (figure 3.1).

The number of endothelial cells present in the corpus luteum exhibited significant variation relative to the stage of the luteal phase (figure 2a; $p<0.05$). The number of cells present in the early luteal phase (117; confidence intervals 97-137 cells per high power field) was significantly lower than in the mid luteal phase (276; confidence intervals 256-296 cells per high power field; $p<0.05$) and late luteal phase (254; confidence intervals 236-272 cells per high power field; $p<0.05$). No significant difference was observed between the number of endothelial cells in the late corpus luteum and that in the hCG stimulated corpus luteum (238; confidence intervals 216-260 cells per high power field; $p=0.17$)

3.3.2 Proliferating cells

Ki67 immunostaining was also observed in all sections examined. The majority of cells exhibiting positive immunostaining were endothelial cells (figure 3.1).

The number of proliferating cells of all types present in the corpus luteum, as judged by positive Ki67 staining, exhibited significant variation relative to the stage of the luteal phase ($p<0.05$). The number present in the early luteal phase (248, confidence intervals 168-328 cells per high power field) was significantly higher than that in the mid-luteal phase (43; confidence intervals 29-57 cells per high power field; $p<0.05$) and late luteal phase (53, confidence intervals 40-66 cells per high power field; $p<0.05$). No significant difference was observed between the total number of proliferating cells in the late corpus luteum and that in the hCG stimulated corpus luteum (78; confidence intervals 55-101 cells per high power field; $p=0.26$).

3.3.3 Proliferating endothelial cells

The number of endothelial cells present which were positive for Ki67 also exhibited significant variation relative to the stage of the luteal phase (figure 2b). The number present in the early luteal phase (120, confidence intervals 98-142 cells per high power field) was significantly higher than in the mid-luteal phase (24, confidence intervals 10-38 cells per high power field; $p<0.05$), and late luteal phase (30, confidence intervals 21-39: $p<0.05$). No significant difference was observed between the number of proliferating endothelial cells in the late corpus luteum and that in the hCG stimulated corpus luteum (47, confidence intervals 33-61 cells per high power field; $p=0.49$).

3.3.4 Proliferating non-endothelial cells

The total number of proliferating cells of non-endothelial origin followed a similar pattern, exhibiting significant variation relative to the stage of the luteal phase (figure 2c; $p<0.05$). The number present in the early luteal phase (128, confidence intervals 70-166) was significantly higher than that in the midluteal phase (19, confidence intervals 15-23; $p<0.05$) and late luteal phase (22, confidence intervals 19-25; $p<0.05$). No significant difference was observed

between the number of proliferating non-endothelial cells in the late corpus luteum and that in the hCG stimulated corpus luteum (31, confidence intervals 21-41 cells per high power field; p=0.38).

3.3.5 Proliferating steroidogenic cells

Proliferating steroidogenic cells were rare, making up less than 5% of total proliferating cells (figure 3.3). There was no significant variation in total number of proliferating steroidogenic cells throughout the luteal phase or in simulated early pregnancy.

3.4 Discussion

3.4.1 General points

This study demonstrates that cell proliferation follows the mid-cycle LH surge and continues throughout the luteal phase, but simulated early pregnancy in women is not associated with a further increase. Proliferating cells were present at all stages of the luteal phase and during luteal rescue. The largest sub-group of proliferating cells were endothelial. Dual immunostaining of 3βHSD and Ki67 demonstrated that very few of the proliferating non-endothelial cells were steroidogenic. Morphological studies have demonstrated that during formation of the corpus luteum endothelial cells grow inwards from the stroma to supply the steroidogenic cells of the gland (Zheng et al 1993). After this initial phase of growth endothelial cell proliferation has been shown to continue at a lower level, falling further when the corpus luteum regresses (McClure et al 1994). These results are concordant with those of a recent report in the rhesus monkey, in demonstrating a high degree of endothelial cell proliferation as the corpus luteum forms (Christenson and Stouffer 1996). The observation of proliferation in non-endothelial cells has not been previously described in the human corpus luteum. Our finding that the majority of these cells are non-

steroidogenic is supported by the low incidence of mitotic figures observed in steroidogenic cells of the corpus luteum (Corner 1956).

3.4.2 Angiogenesis during simulated early pregnancy

Proliferation of luteal cells was examined in hCG stimulated corpora lutea. The pattern of endothelial cell proliferation in early pregnancy in women has not been previously described. Both the blood flow to and the steroidogenic output from the corpus luteum increase with hCG stimulation (Atkinson et al 1975; Salim et al 1994). However, no increase in either endothelial or non-endothelial cell proliferation was detected during luteal rescue. Increasing blood supply to the gland may be mediated by changes in vascular tone at this stage, with angiogenesis occurring at a later point. Hypertrophy, rather than hyperplasia of luteal cells may account for the early changes in luteal mass.

3.4.3 Suitability of antibodies

Antibodies directed against vWF and Ki67 were used to demonstrate endothelial cells and proliferating cells respectively. vWF is an intrinsic clotting cascade protein and is produced in cells of endothelial lineage. Comparative studies have shown this antibody to be both sensitive and specific in detecting endothelial cells (McComb et al 1982).

Ki67 antibody specifically identifies proliferating cells (Gerdes et al 1991) and is thought to recognise an epitope of a nuclear non-histone protein (Boulton and Hodgson 1995). Comparison studies in a number of tissues have shown Ki67 immunostaining to compare favourably with the gold standard methods of bromodeoxyuridine and radiolabelled thymidine incorporation (Sasaki et al 1988; Kamel et al 1989).

3.4.4 Proliferation throughout the luteal phase

Cell proliferation in the corpus luteum does not appear to be completely limited to any one stage of the luteal phase. Endothelial cell proliferation persists at a lower rate through the mid and late luteal phases, when the functional capacity of the gland is declining, as measured by progesterone and inhibin A production (Illingworth et al 1990). Similarly, there is still a significant rate of non-endothelial cell proliferation at these stages. Other investigators have noted similar levels of cell proliferation in the late luteal phase in human (McClure et al 1994) and rhesus monkey (Christenson and Stouffer 1996). The absence of further growth of the corpus luteum despite apparent continuing cellular proliferation implies that there is a simultaneous process of cell loss. Different forms of cell death in the human and primate corpus luteum have recently been demonstrated (Funayama et al 1996; Fraser et al, 1995). Indeed high levels of programmed cell death have been shown to follow induced luteal regression in primates (Fraser et al 1995; Young et al 1996) while cells with morphology characteristic of apoptosis have been identified in during luteolysis in the sheep (O'Shea et al 1977).

3.4.5 Overview

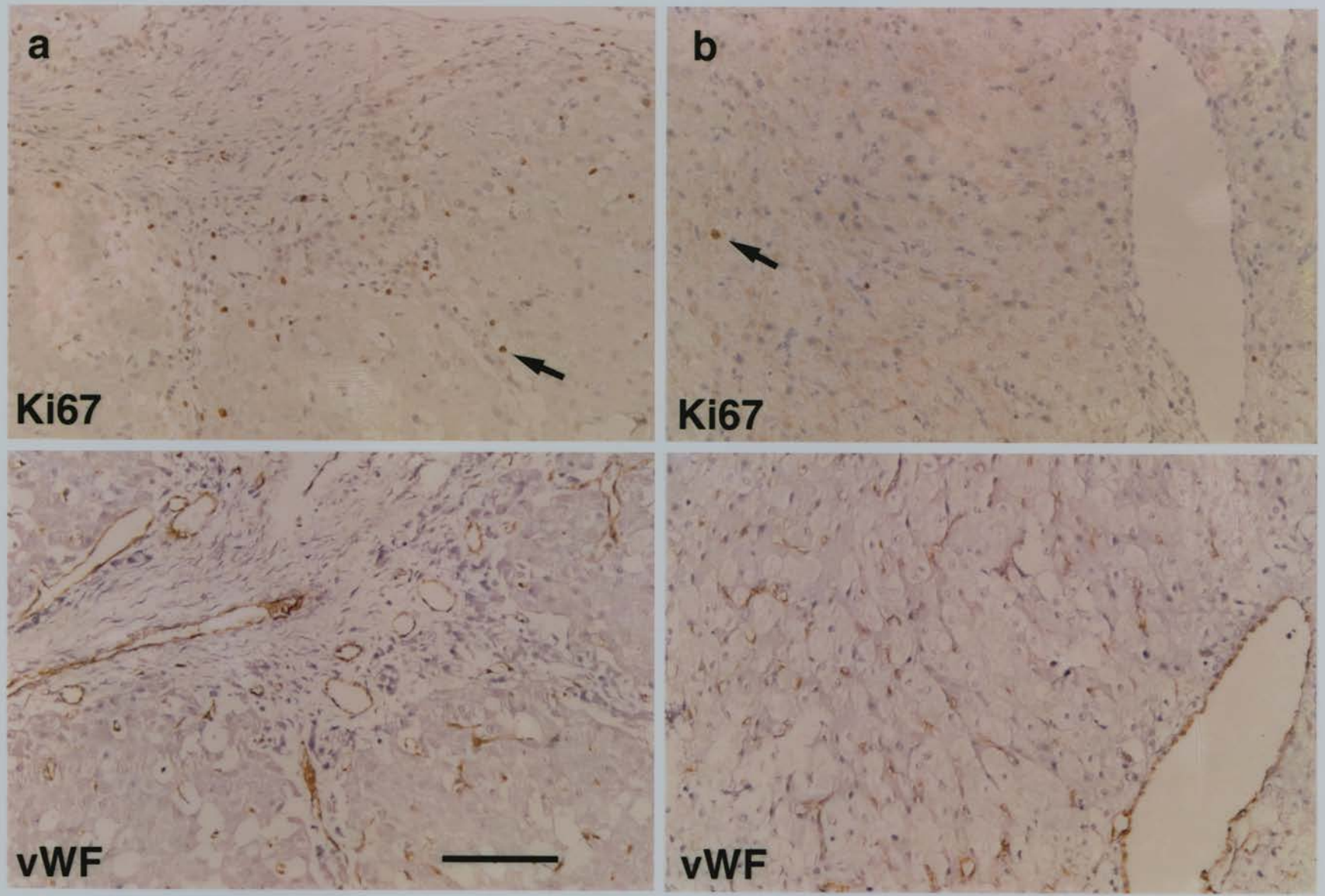
In conclusion, this study has investigated the role of endothelial cell proliferation in luteal rescue analogous to that of early pregnancy. No differences in the rate of proliferation of any cell type were found indicating that the luteal rescue of pregnancy is not due to enhanced rates of cellular proliferation.

Figure 3.1

Photomicrographs of serial sections from early(a), mid (d), late, (c) and rescued corpora lutea (d).

Serial sections are stained for von Willebrand factor (vWF) and Ki67. Arrows indicate Ki67 positive cells.

In each case the scale bar represents 100μm.


a
Ki67
b
Ki67
vWF
vWF

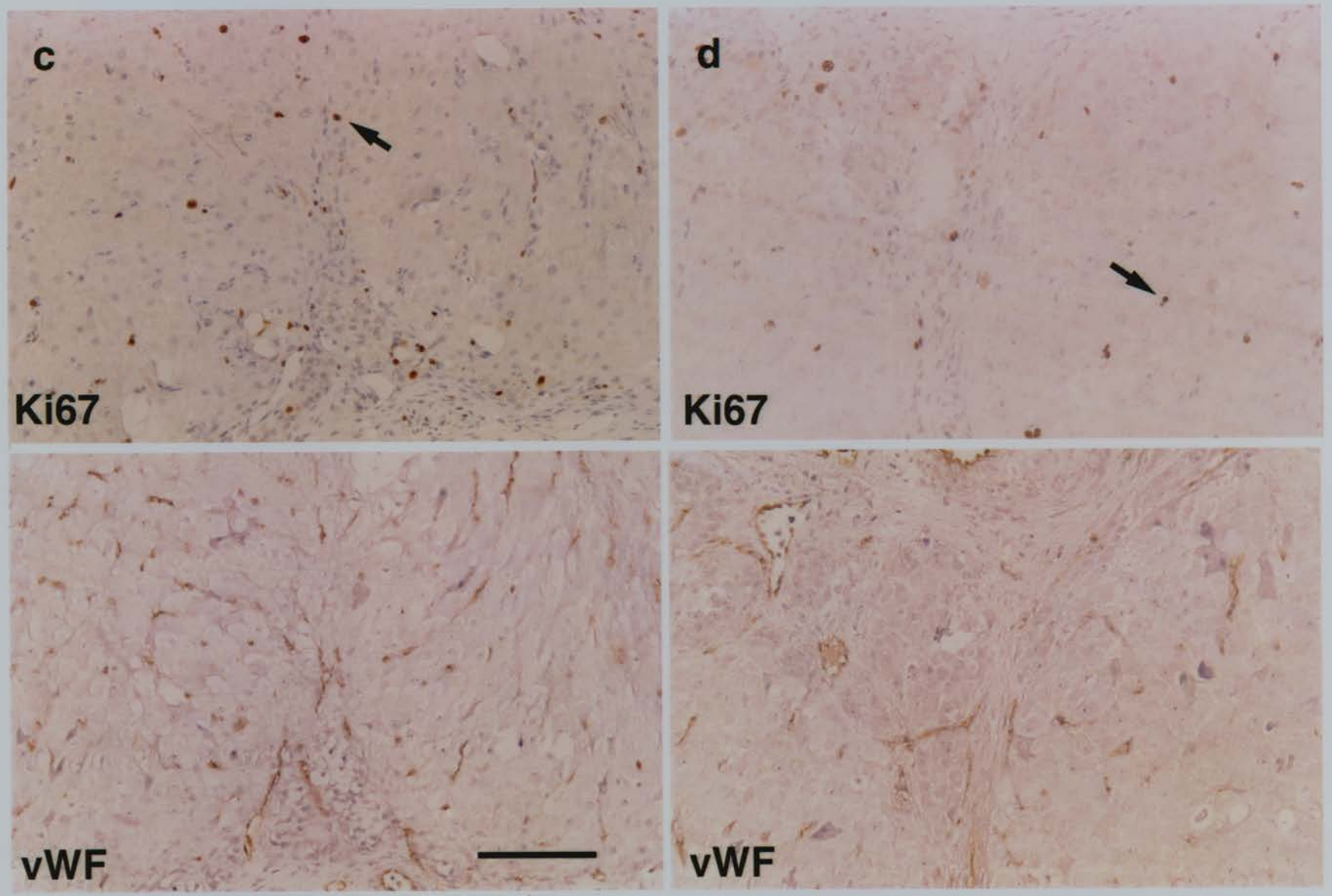

c
Ki67
d
Ki67
vWF
vWF

Figure 3.2

(a) The mean (+/-SE) number of endothelial cells present per high power field at different stages of the luteal phase.

(b) The mean (+/-SE) number of endothelial cells exhibiting proliferation per high power field at different stages of the luteal phase.

(c) The mean (+/-SE) number of non-endothelial cells exhibiting proliferation per high power field at different stages of the luteal phase.

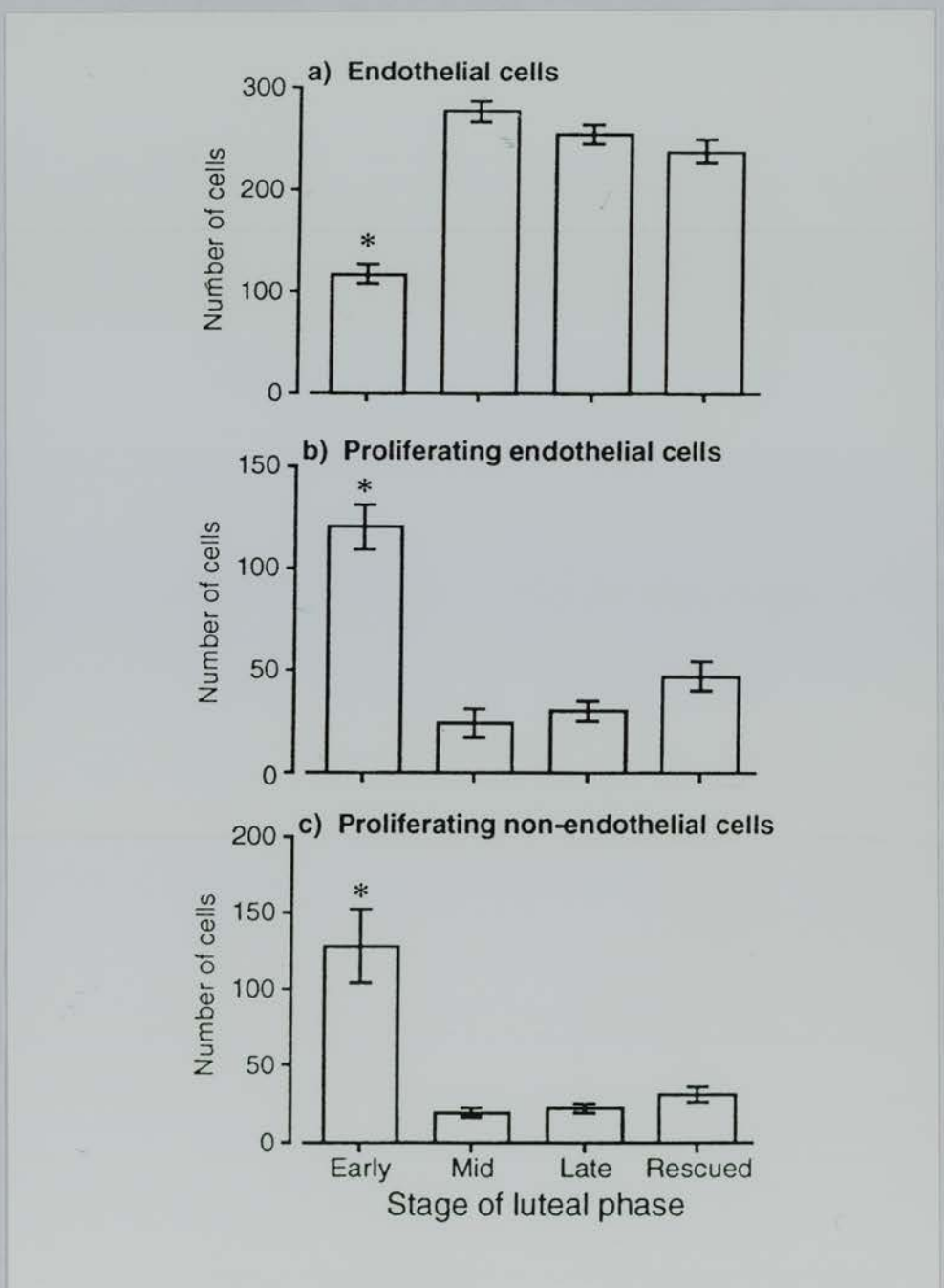
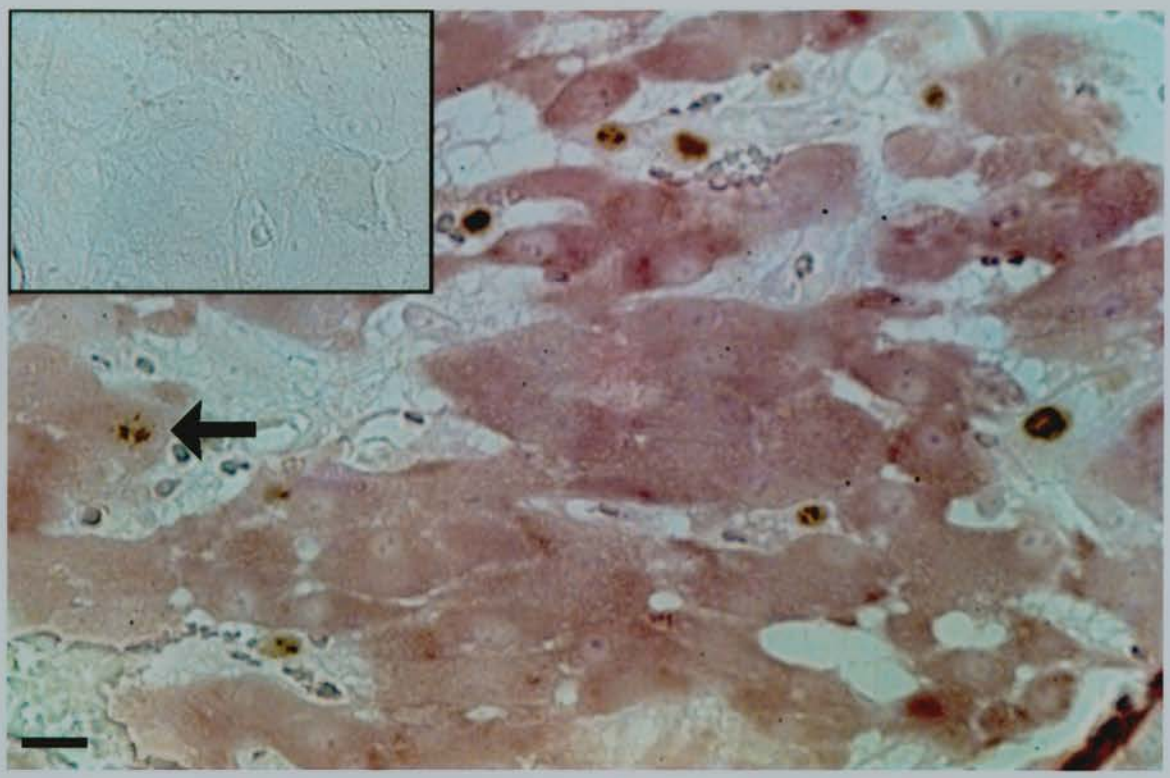


Figure3.3

Photomicrograph of early luteal phase tissue with dual immunostaining for Ki67 (brown staining) and 3βHSD (red staining). Insert shows negative control immunostaining. The arrow indicates a cell which exhibits positive immunostaining for both Ki67 and 3βHSD.

The scale bar represents 20μm.



CHAPTER FOUR

ANGIOGENIC GROWTH FACTORS IN THE HUMAN CORPUS LUTEUM

4.1 Introduction

4.1.1 Vascular factors and luteal function

Vascular factors may be important for the regulation of luteal function. The corpus luteum is a highly vascular structure with a blood flow which is, per unit weight, the highest of any tissue in the body (Reynolds et al 1992; Bruce and Moor 1972; Bruce et al 1984; Wiltbank et al 1988). A dense network of luteal vessels develops during the first three days after ovulation, and grows from the theca lutein cell layer to supply the previously avascular granulosa lutein cells (Corner 1956; Zheng et al 1994). Proliferation of endothelial cells in the human corpus luteum is maximal as the corpus luteum forms following the LH surge. (Rodger et al 1997, see chapter 3). Although Ghinea et al (1994) have described the presence of LH binding sites in endothelial cells in the rat ovary, Duncan et al noted in 1996 that human luteal endothelial cells did not express LH receptor messenger RNA. In order for LH to trigger this angiogenesis, a paracrine signal must pass between the LH receptor-positive steroidogenic cells and the endothelial cells of the gland. Local secretion of vascular growth factors may thus account for the high rate of angiogenesis observed during establishment of the corpus luteum.

4.1.2 Vascular factors in pregnancy

Proliferation of endothelial cells does not change during hCG simulated early pregnancy (Rodger et al 1997; Christenson and Stouffer 1996). However it has been demonstrated that the blood flow to the corpus luteum increases during early pregnancy (Baber et al 1988; Salim et al 1994; Wiltbank et al 1989),

allowing the delivery of substrates necessary for progesterone biosynthesis. Luteal blood flow may initially be increased by changes in vascular tone, with blood vessel growth occurring at a later stage. Upregulation of growth factor expression may be detectable at an earlier stage than increased rates of endothelial cell proliferation.

4.1.3 Angiogenic growth factors

Bovine, ovine and porcine ovaries produce angiogenic factors throughout the luteal phase and in early pregnancy (Reynolds et al 1992; 1994; Ricke et al 1995; Grazul-Bilska et al 1992). Analysis of ovine and bovine luteal extracts has led to the identification of two heparin-binding angiogenic growth factors (Grazul-Bilska et al 1993;1995; Doraiswamy et al 1995a;b).

4.1.4 Basic fibroblast growth factor (bFGF)

bFGF promotes angiogenesis by acting through membrane receptors to increase the rate of endothelial cell proliferation (Bagavandross and Wilkes 1991). In other tissues, bFGF and its receptors are localised in the endothelial cell compartment (Bikfalvi et al 1997). bFGF activity has been detected in bovine corpora lutea (Grazul-Bilska et al 1993) and bFGF has been localised in bovine endothelial and steroidogenic luteal cells (Schams et al 1994). In addition, bFGF receptors 1 and 2 have been immunolocalised to both endothelial and steroidogenic cells of bovine corpora lutea (Doraiswamy et al 1995b; Redmer and Reynolds 1996).

4.1.5 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) stimulates angiogenesis by increasing endothelial cell mitosis and vascular permeability (Ferrara and Davis-Smith 1997; Roberts et al 1995), and acts in synergy with bFGF (Stavri et al 1995). Increased vascular permeability allows fibrin deposition providing a

framework for blood vessel formation. Although previous studies have localised VEGF to perivascular cells in domestic species (Doraiswamy et al 1996) VEGF has been immunolocalised in steroidogenic luteal cells in primates (Kamat et al 1995; Gordon et al 1996). VEGF has been shown to act through two tyrosine kinase linked receptors, flt and kdr, which are present on endothelial cell membranes in other tissues (Ferrara et al 1997). Fluctuations in levels of these growth factors or their receptors may affect the rate of luteal angiogenesis which may in turn influence luteal function.

4.1.6 Regulation of growth factors by the hypothalamic-pituitary axis.

VEGF secretion by the corpus luteum may be regulated by the hypothalamic-pituitary axis in primates, as administration of GnRH antagonist to rhesus monkeys has been shown to result in a decrease in VEGF mRNA expression (Ravindranath et al 1992b). Although hCG induced VEGF secretion has been demonstrated in luteinised granulosa cells in vitro (Neulen et al 1995), and hCG administration has been shown to increase urinary VEGF output in women (Roberts and Palade 1995) the distribution of these growth factors in the corpus luteum of early pregnancy or during luteal regression in women is unknown.

4.1.7 Aims of this chapter

This chapter aims to determine whether production of VEGF or bFGF vary according to luteal function. Immunohistochemistry, protein electrophoresis and in situ hybridisation studies were utilised to investigate whether the distribution or localisation of these factors changed throughout the luteal phase or in simulated early pregnancy.

4.2. Methods

4.2.1 Immunohistochemistry

Sections (5μm) were cut, air-dried at 56°C, dewaxed in xylene then rehydrated in 100%, 95%, and 70% ethanol. Sections were exposed to three 5 minute cycles of microwave irradiation at 700w, in citrate buffer 0.01M, pH 6.0 (Shi et al 1993). Sections were preabsorbed for 20 minutes with normal goat serum (SAPU, Carluke, UK) diluted 1:5 in tris buffered saline. The primary antibody, a polyclonal antibody raised in rabbit against human VEGF protein (Santa Cruz, Santa Cruz, CA, USA) was applied at 1:100 dilution (0.5 mg/mL protein concentration). For control sections, 0.5 mg/mL of rabbit serum (Dako, High Wycombe, UK) was used in place of primary antibody. After incubation for 20 hours at 4°C, the secondary antibody, biotinylated goat anti rabbit (Dako) was applied at a dilution of 1:500 for 45 minutes. Colouring was obtained with Vector Red chromogen (Vector, Peterborough, UK) by means of a reaction with avidin covalently coupled to alkaline phosphatase (Dako).

In order to identify the nature of cells staining positively for VEGF, sections serial to those immunostained for VEGF were immunostained for P450C17. This enzyme is specifically localised in theca lutein cells (Sasano et al 1989). Sections were blocked with normal swine serum diluted 1:5 in tris buffered saline. Polyclonal 17-alpha hydroxylase cytochrome P450 antibody, raised in rabbit, (gift of Prof M Waterman, Vanderbilt University, Nashville), was applied at 1:750 dilution, and sections were incubated at 4°C for 20 hours. Biotinylated swine anti-rabbit secondary antibody (Dako) at a dilution of 1:500 was then applied for 30 minutes. Colouring was obtained with nitroblue tetrazium chloride (NBT) by means of a reaction with avidin covalently coupled to alkaline phosphatase.

Control sections were incubated with normal rabbit serum (Dako) at 1:750 dilution in place of 17-alpha hydroxylase primary antibody.

The intensity of immunostaining for VEGF at different stages of luteal function was assessed in four low power (x100) fields from opposite quadrants of the glandular component of the section. Staining was graded as follows: 0= no staining; 1= weak staining; 2= moderate staining; 3= deeper immunostaining, 4= intense immunostaining (figure 2). In all cases the observer was unaware of the nature of the section being examined. Differences in the intensity of staining were investigated by one-way analysis of variance using stage of the luteal phase as a between subject (corpus luteum) variable. The intensity of immunostaining in late and rescued corpora lutea was also compared in the same manner.

Immunohistochemistry for bFGF was carried out using the same methodology as for VEGF using a mouse monoclonal antibody directed against human bFGF (Upstate Biotech, Madison, NY, USA) at a dilution of 1:100 (5mg/mL). Negative control sections were incubated with mouse IgG at a 5mg/mL concentration in place of the primary antibody. After application of rabbit anti-mouse biotinylated secondary antibody staining was visualised by means of a reaction with avidin covalently coupled to horseradish peroxidase (DAKO).

4.2.2 SDS-PAGE and immunoblotting

Protein was extracted from eight corpora lutea (2 early, 2 mid, 2 late and 2 rescued). Tissue was solubilised in 0.1% SDS buffer at 4^{o}C, sonicated and centrifuged for 5 minutes. Supernatant was stored in aliquots at -20^{o}C prior to use. Protein content in the homogenates was determined by Bradford's method using a commercial kit (Bradford 1976), (Biorad, Hercules, CA, USA). For polyacrylamide gel electrophoresis (PAGE), 200μg samples were denatured by boiling for five minutes in an equal volume of sample buffer containing 5% β-mercaptoethanol and run on an 11% polyacrylamide gel alongside molecular weight markers (Rainbow markers, Biorad). Gels were hybridised to a nitrocellulose membrane (Hybond C, Amersham International,

Buckinghamshire, UK) for 18 hours at 50 volts. Western blots obtained were analysed for (1) VEGF protein with polyclonal VEGF antibody at 1:500 dilution and (2) bFGF using monoclonal bFGF antibody at a dilution of 1:500. Proteins were detected with diaminobenzidene by means of a reaction with avidin covalently coupled to horseradish peroxidase (Dako).

4.2.3 In situ hybridisation

In order to confirm the distribution of VEGF staining, in situ-hybridisation studies for VEGF and also for flt, a VEGF receptor, were undertaken.

For in situ hybridisation, radial segments from eight corpora lutea, and also from human term placenta (to be used as a positive control tissue) were frozen in mounting medium as described in chapter 2 and stored at -70 ^{o}C. Frozen sections (5μm) were attached to sterile TESPA coated glass slides, and fixed in 4% paraformaldehyde for 5 minutes. The VEGF and flt probes (Sharkey et al 1993; Cooper et al 1995) were generated from a 753 base and 272 base pair fragments respectively in pcDNAIII plasmid (Promega, Madison, WI, USA). To obtain antisense and sense probe templates the plasmid was lysed by EcI-xIX and Xhol restriction endonuclease digestion (Boehringer Mannheim, Mannheim, Germany) and purified. Antisense and sense labelled riboprobes for VEGF were synthesised via incorporation of ^{35}S-UTP (Amersham International) by addition of T3 or T7 polymerases (Promega). Similarly, antisense and sense flt probes were synthesised by addition of T7 or T3 polymerases respectively. The probes were then treated with ribonuclease-free DNase1 (Promega), and hybridisation was carried out at 55^{o}C for 20 hours. Sections were subjected to washes of increasing stringency, culminating in a high stringency wash for 30 minutes at 70^{o}C in a solution containing 2mM dithiotreitol and 0.1M standard saline citrate. Autoradiography was carried out using NTB emulsion (Kodak, Rochester, NY, USA). The emulsion coated slides were stored at 4^{o}C with

desiccant for an exposure time of 6 weeks. The slides were developed in Kodak D19 developer, counterstained with haematoxylin and mounted.

4.3 Results

4.3.1 Immunohistochemistry

Immunostaining for VEGF was identified in all corpora lutea examined (figure 4.1). Immunostaining was localised in theca and granulosa lutein cells throughout the luteal phase and in simulated early pregnancy. In each case, staining of theca lutein cells was of higher intensity than that of granulosa lutein cells. Immunostaining for VEGF was present in all theca lutein cells of the corpora lutea at all stages of the luteal phase and in simulated early pregnancy. Granulosa lutein cells stained homogeneously for VEGF in the early, mid and late luteal phases, but staining became patchy in hCG treated corpora lutea, where individual granulosa lutein cells stained strongly for VEGF while adjacent cells were only weakly positive. Overall intensity of immunostaining for VEGF did not vary significantly between different stages of luteal function (figure 4.2). Endothelial cells did not exhibit immunostaining for VEGF at any stage of the luteal phase.

Immunostaining for bFGF was in contrast localised in endothelial cells of a minority of luteal blood vessels (figure 4.3). No immunostaining for bFGF was detected in steroidogenic luteal cells. Vessels with positive bFGF immunostaining were located at the periphery of the gland and had muscular walls.

4.3.2 SDS-PAGE and Immunoblotting

A 40kDa protein band, consistent with VEGF (Ferrara and Davis-Smith 1997)), was observed at all stages of luteal function. The intensity of the band did not change throughout the luteal phase or in simulated early pregnancy (figure 4.4).

An 18kDa protein band, consistent with bFGF (Bikfalvi et al 1997), was detected at all stages of luteal function. The intensity of the band did not change throughout the luteal phase or in simulated early pregnancy. A larger 24kDa band, consistent with a nuclear form of bFGF (Ferrara and Davis-Smith 1997) was similarly detected at all stages of luteal function (figure 4.5).

4.3.3 In situ-hybridisation

Hybridisation of antisense VEGF probe to tissue sections was localised predominantly in theca lutein cells. No such hybridisation was evident in control sections incubated with sense riboprobe (figure 4.6).

In positive control tissue VEGF antisense hybridisation was present in syncytiotrophoblast as previously reported (Shiraishi et al 1996). No such hybridisation was observed with sense riboprobe (figure 4.7).

Hybridisation of antisense probe to flt, a VEGF receptor, was localised in endothelial cells of luteal blood vessels in the corpus luteum. No such hybridisation was detected with sense riboprobe (figure 4.8).

4.4 Discussion

4.4.1 General points

This is the first study to examine distribution of the angiogenic factors VEGF and bFGF throughout the lifespan of the human corpus luteum and represents the first description of bFGF localisation in the human corpus luteum. VEGF was specifically localised in steroidogenic cells of the corpus luteum, while bFGF was localised in endothelial cells of luteal blood vessels.

This localisation fits with a role in regulation of the luteal vasculature. Luteal maintenance is associated with increased blood flow and is triggered by hCG secreted from the feto-placental unit (Redmer and Reynolds 1996). hCG may induce changes in blood flow to the corpus luteum by influencing local production of paracrine regulators of angiogenesis such as VEGF from

steroidogenic cells (Neulen et al 1995). Autocrine secretion of growth factors such as bFGF may also be important for luteal angiogenesis.

4.4.2 VEGF localisation

A previous study localising VEGF in the human corpus luteum has reported that this growth factor is localised predominantly in the granulosa lutein cells, with theca lutein cells containing smaller amounts of VEGF protein (Kamat et al 1995). The current work demonstrates in a wide range of luteal tissue that both VEGF protein and mRNA are present at higher levels in theca than in granulosa lutein cells. Blood vessels initially grow into the developing corpus luteum from invaginations of theca cell rich connective tissue (Corner 1956; Zheng et al 1994). The high level of angiogenesis observed (McClure et al 1994) in these areas may be due to thecal VEGF production.

Controversy already exists concerning VEGF localisation with the human antral follicle. Kamat et al (1995) describe VEGF in granulosa cells of ovarian follicles, while Gordon and co-workers localised VEGF immunostaining to the theca cell compartment. It is possible that these inconsistencies are due to the existence of alternatively spliced variants of VEGF. Granulosa and theca lutein cells may produce different relative quantities of the VEGF forms. Certain antibodies may not recognise all forms of VEGF, leading to a particular pattern of staining. The VEGF antibody used in this study was polyclonal and was directed against a 21 amino-acid sequence from the N-terminal of VEGF, a region common to all forms of the protein.

The localisation of VEGF to theca cells of the corpus luteum described above is confirmed by in situ hybridisation using a probe common to all VEGF splice variants. In addition, SDS-PAGE and immunoblotting validates that the antibody used detects a protein of a size consistent with VEGF.

4.4.3 VEGF and changing luteal function

Levels of VEGF production did not change throughout the luteal phase or in simulated early pregnancy. In contrast to in vitro studies, hCG administration did not increase VEGF production. VEGF may be of importance in maintaining the luteal vasculature during the lifespan of the gland, but is unlikely to be involved in regulation of luteal function.

In situ hybridisation studies localised flt, a VEGF receptor, in endothelial cells of small vessels within luteal tissue. This distribution is consistent with a paracrine mode of action for VEGF in the corpus luteum. Varying expression of the VEGF receptors flt and kdr may regulate the angiogenic potential of the gland at different stages of luteal function. Further work is required to elucidate the distribution and level of expression of these receptors throughout the lifespan of the corpus luteum and in simulated early pregnancy.

4.4.4 bFGF localisation

In contrast to VEGF distribution, bFGF was immunolocalised in endothelial cells of peripheral luteal blood vessels. In other tissues bFGF is also present in endothelial cells, and the bFGF receptors are localised on endothelial cell membranes, suggesting an autocrine role for this growth factor. In addition, bFGF receptors have been identified on steroidogenic as well as endothelial cells in the bovine corpus luteum. The effects of bFGF on steroidogenic cell function are unknown. Further work is required to establish the localisation of the bFGF receptor in the human corpus luteum.

4.4.5 bFGF and changing luteal function.

Immunoblotting detected both nuclear and cytoplasmic forms of bFGF protein at all stages of luteal function and in simulated early pregnancy. Consistent levels of bFGF production during luteal maintenance and regression imply that

this growth factor is not directly involved in the control of luteal function. bFGF may instead play a role in the maintenance of the vasculature of the corpus luteum throughout its lifespan. As previously discussed for VEGF, changes in bFGF receptor number or distribution may be implicated in regulating luteal function.

4.4.6 Overview

In summary, VEGF is produced by theca and granulosa lutein cells in the human corpus luteum throughout the luteal phase and in simulated early pregnancy. Flt, a VEGF receptor, is located on endothelial cells of luteal blood vessels. VEGF may act in a paracrine manner to maintain the vasculature of the corpus luteum throughout its lifespan. Unchanging production of VEGF during luteal regression and maintenance suggest that this growth factor is not a direct regulator of luteal lifespan.

Localisation of bFGF in endothelial cells is consistent with an autocrine role for this growth factor in the maintenance of the luteal vasculature. Consistent bFGF production throughout the luteal phase and in simulated early pregnancy suggests that secretion of this growth factor does not directly regulate luteal function.

Figure 4.1

The bar in each case represents 50μm

Immunostaining for VEGF in early, mid, late and rescued corpora lutea. VEGF immunostaining is of greater intensity in theca lutein (T) than in granulosa lutein cells (G). The more intense thecal staining corresponds to immunostaining for P450C17 which is specific to theca cells (b) (brown immunostaining). Intense thecal staining for VEGF is evident in a high power view (a). Inserts show negative control sections, incubated with non-immune serum in place of primary antibody, and are free from staining in each case.

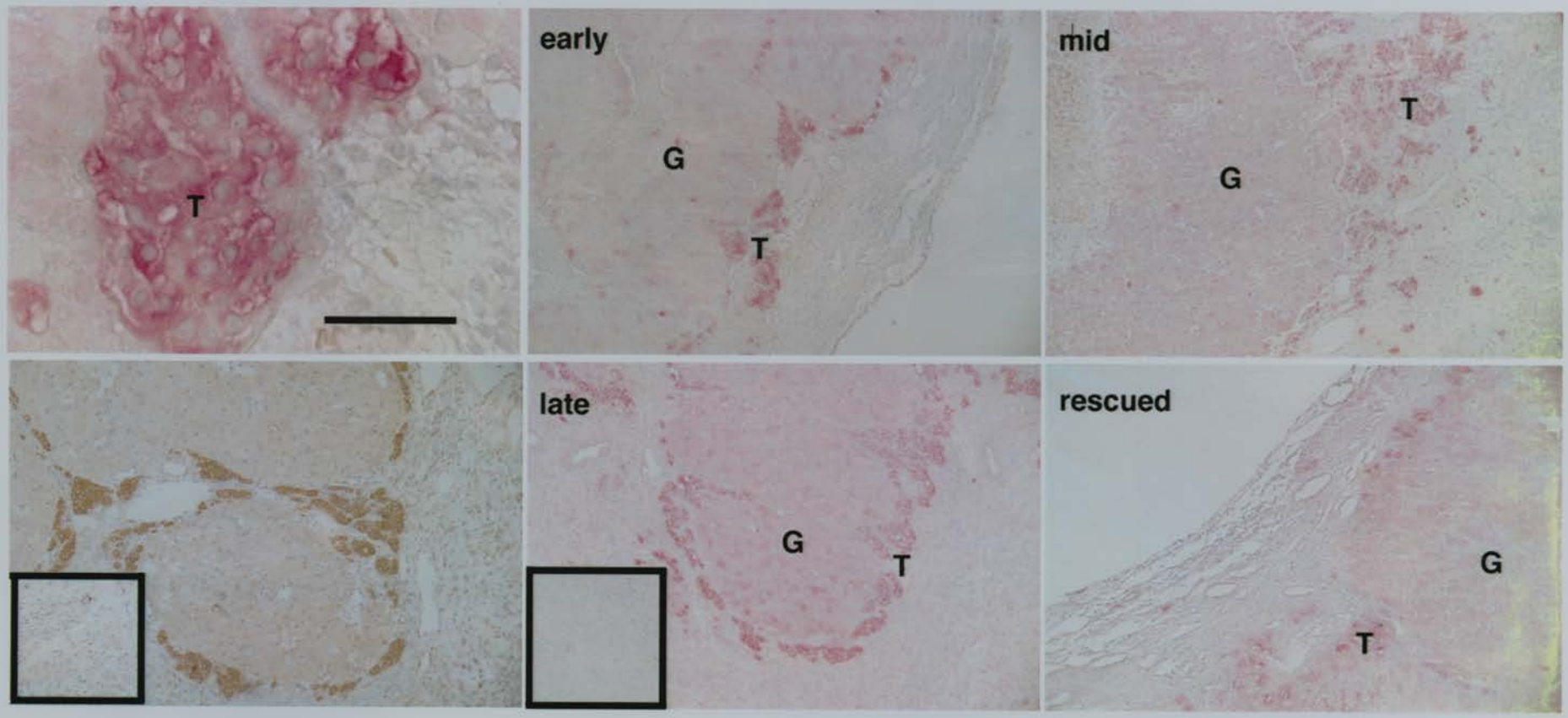

T
early
G
T
mid
T
G
late
G
T
rescued
G
T

Figure 4.2

Graphical representation of intensity of VEGF immunostaining in theca lutein (solid) and granulosa lutein (shaded) cells throughout the luteal phase and in simulated early pregnancy. At all stages immunostaining for VEGF was more intense in theca than in granulosa cells; immunostaining intensity did not change significantly with changing luteal function.

Immunostaining for VEGF throughout the luteal phase

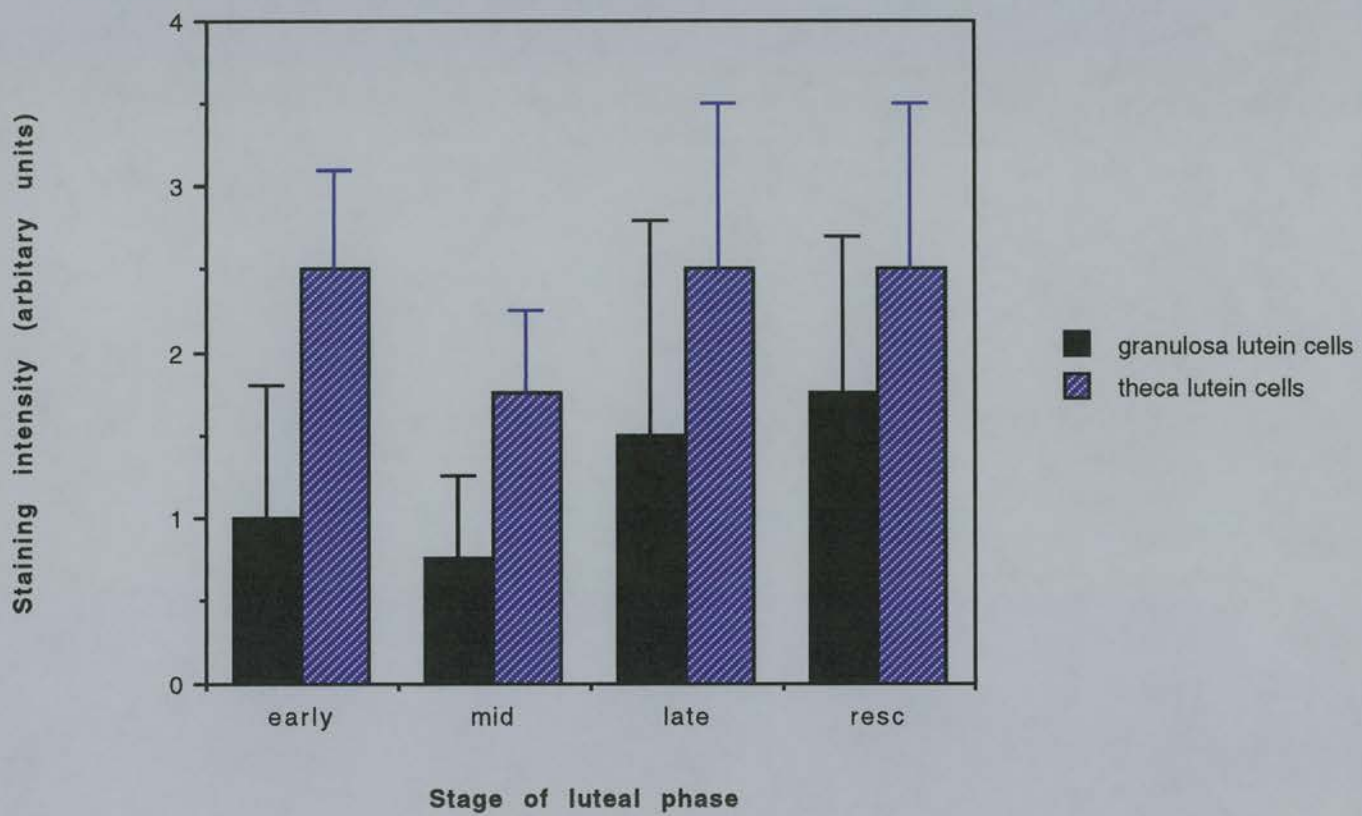


Figure 4.3

Immunostaining for bFGF in a section of late luteal phase corpus luteum (a). Immunostaining is localised in a small blood vessels with muscular walls at the periphery of the gland. The scale bar represents 50μm. Figure (b) shows a negative control section, incubated with non-immune serum in place of primary antibody, and is free from staining.

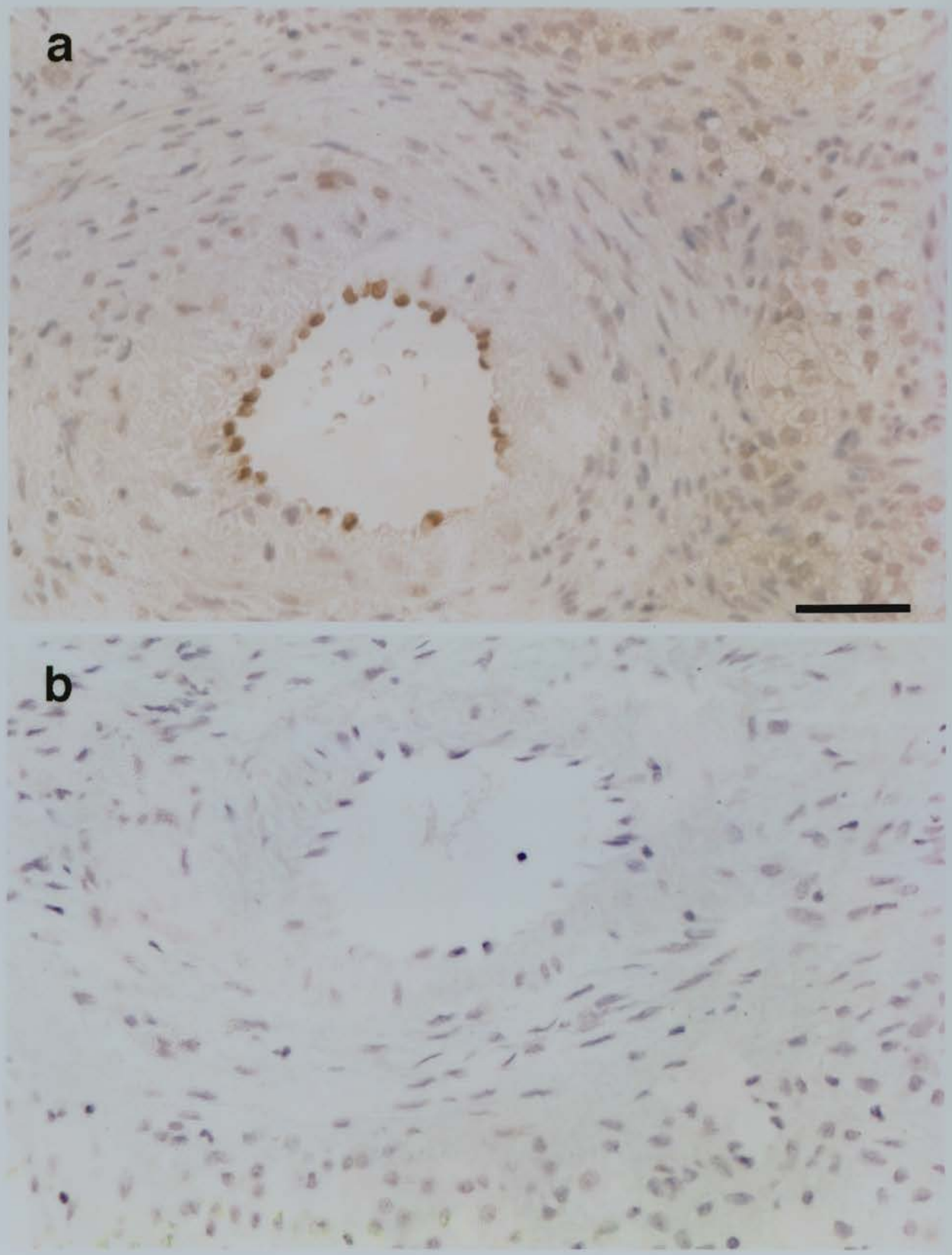

a
b

Figure 4.4

SDS-PAGE and immunoblotting for VEGF in the human corpus luteum. Samples are denoted as follows: E- early, M-mid, L-late, R-rescued corpora lutea. A single protein band was identified at 40kDa consistent with VEGF. Larger bands are due to non-specific staining of albumin and IgGs.

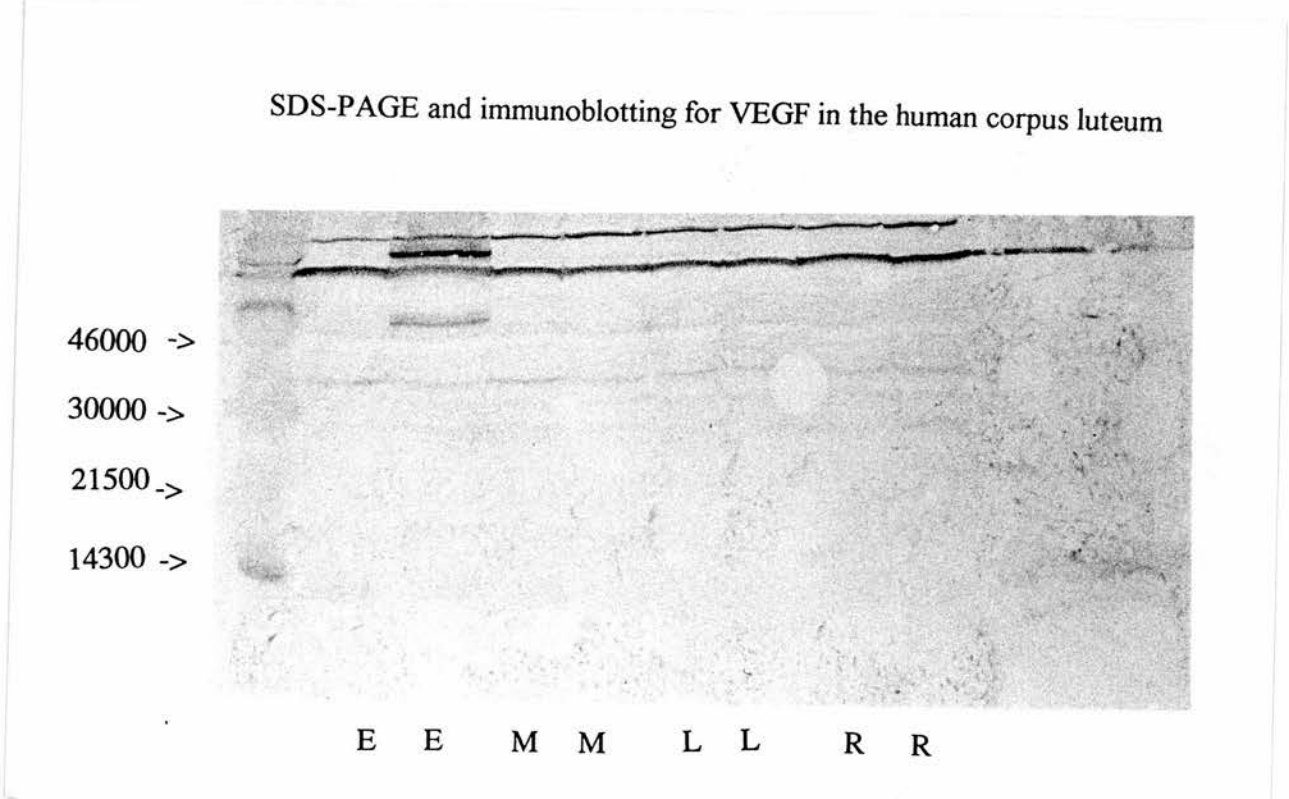


Figure 4.5

SDS-PAGE and immunoblotting for bFGF throughout the luteal phase. Protein samples are denoted as follows; E-early, M-mid, L-late, R- rescued luteal. Protein bands were visible at 18 and 24 kDa, consistent with previously described cytoplasmic and nuclear forms of bFGF.

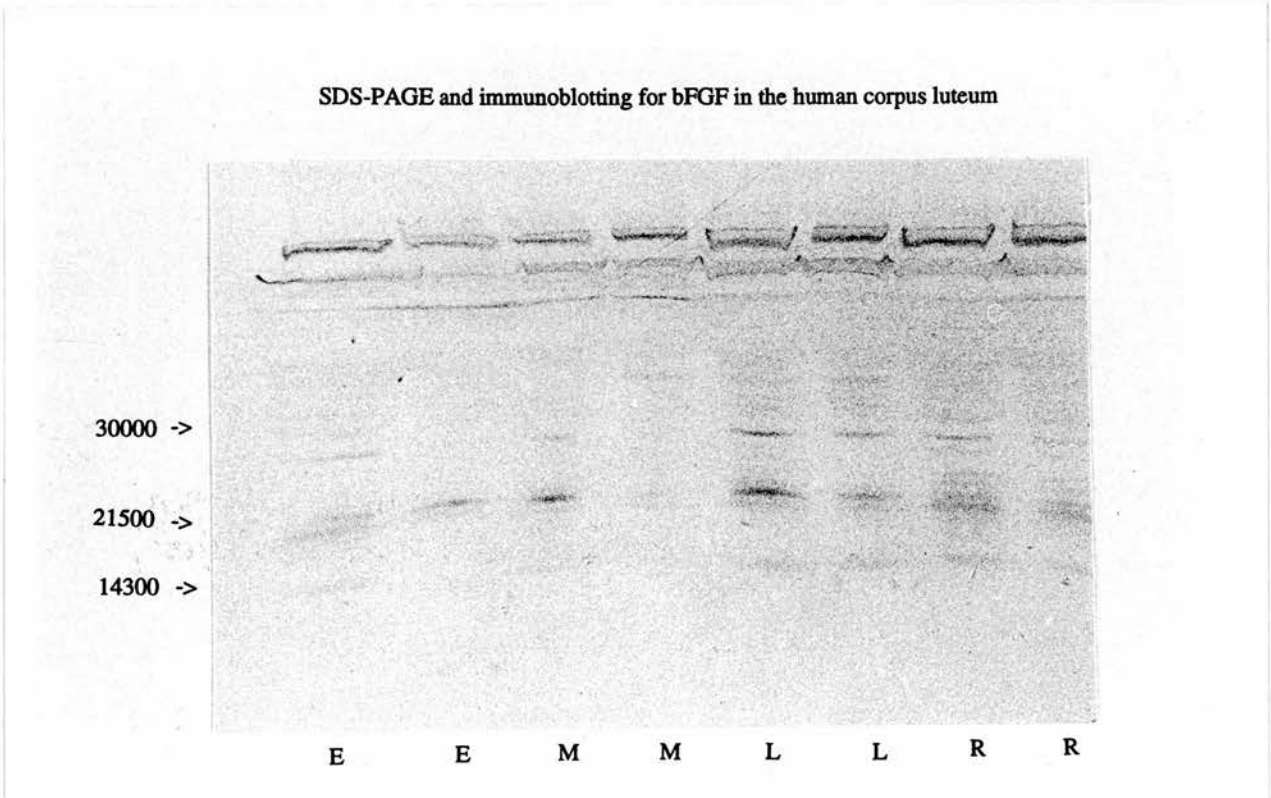


Figure 4.6

In situ hybridisation for VEGF in corpus luteum from the early luteal phase. Top section shows hybridisation of antisense riboprobe under dark field illumination; middle section shows the same section under light field illlumination; lower plate illustrates binding of sense riboprobe under dark field illumination in a negative control section. The scale bar represents 25μm.

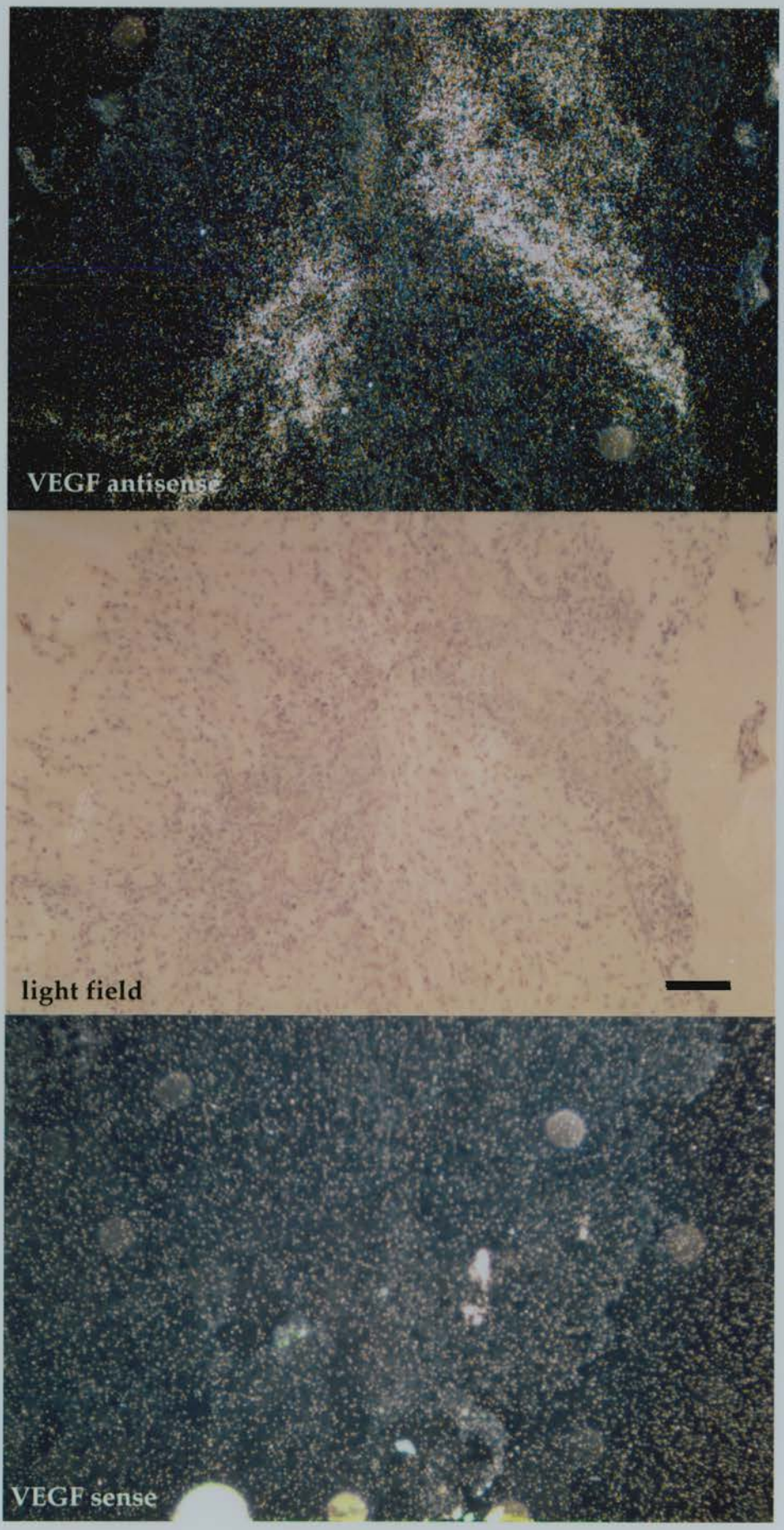

VEGF antisense
light field
VEGF sense

Figure 4.7

In situ hybridisation for VEGF in placenta (positive control tissue). Top section shows hybridisation of antisense riboprobe under dark field illumination; middle plate shows the same section under light field illlumination; lower plate illustrates binding of sense riboprobe under dark field illumination in a negative control section. The scale bar represents 25μm.

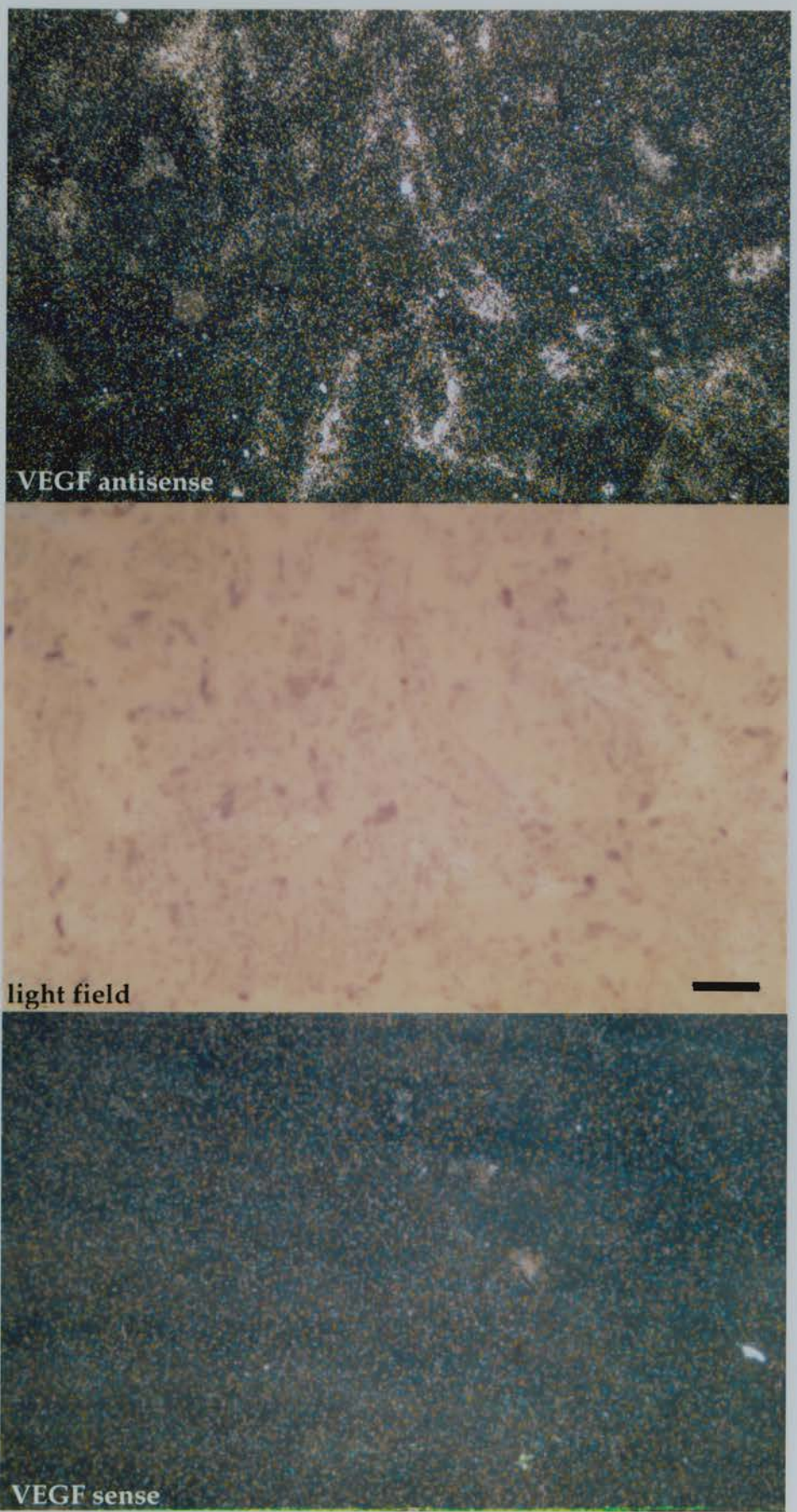
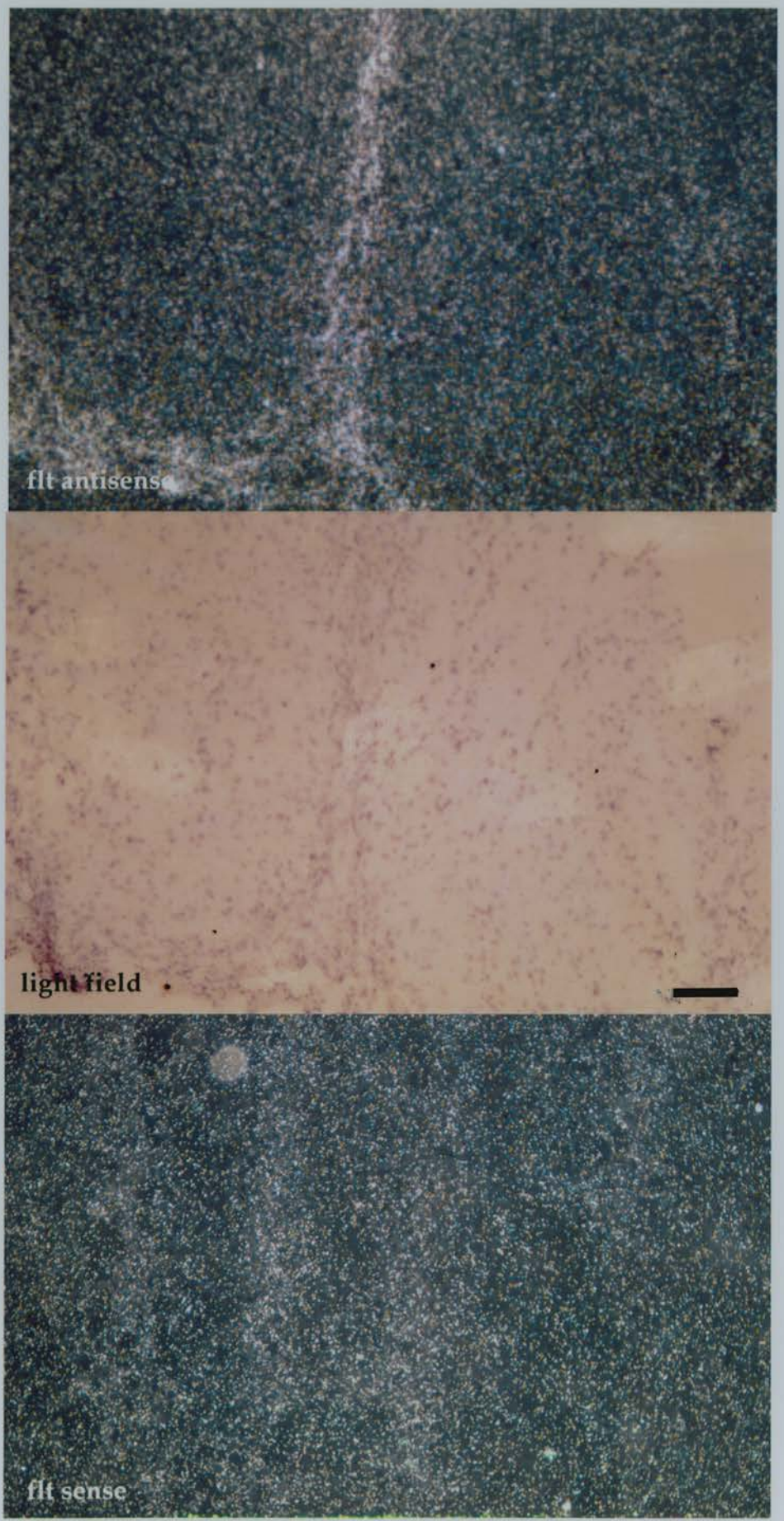

VEGF antisense
light field
VEGF sense

Figure 4.8

In situ hybridisation for flt, a VEGF receptor in corpus luteum from the early luteal phase. Hybridisation with antisense probe is localised in luteal blood vessels, top plate; middle plate shows the same section under light field illlumination; lower plate illustrates binding of sense riboprobe under dark field illumination in a negative control section. The scale bar represents 25μm.


flt antisense
light field
flt sense

CHAPTER 5

CONTROL OF APOPTOSIS IN THE HUMAN CORPUS LUTEUM

5.1 Introduction

5.1.1 Cell death and luteal regression

During the process of luteolysis, the corpus luteum undergoes a loss of function and structure over a short period of time. Recent studies suggest that cell death may be involved in luteal regression. Different forms of cell death including necrosis and autophagocytosis (Fraser et al 1995) and apoptosis (Shikone et al 1996; Young et al 1996; Modlich et al 1996) have been described during luteolysis. A high rate of cell death at the end of the luteal phase could account for the rapid diminution in the function and structure of the gland which is observed at this time. A high rate of cell death may cancel out the continuing proliferation observed in the corpus luteum during functional regression (Rodger et al 1997). The presence of hCG in early pregnancy may result in reduced rates of luteal cell death which may allow luteal growth without increased proliferation as:

Rate of cell accumulation (tissue growth) = rate of cell proliferation - rate of cell death. (Reed 1994).

5.1.2 Apoptosis

There is mounting evidence to suggest that programmed cell death may be the principal form of cell death involved in luteal regression. Apoptosis is a form of physiological cell death characterised by morphological as well as biochemical criteria. The cell shrinks and becomes denser while chromatin becomes pyknotic and marginated in smooth masses against the nuclear membrane (Manjo and Joris 1995; Kerr et al 1972). In contrast to necrosis, organelles do not swell. The nucleus may also break up and the cell may form buds

containing fragments of the nucleus. The buds break away to become membrane bound dense apoptotic bodies which are then phagocytosed by tissue macrophages or neighbouring cells. The time course of apoptosis is swift, with the morphological process taking between 30 minutes and one hour to complete. Biochemically, the DNA of a cell undergoing apoptosis is broken down into segments which are multiples of 200 base pairs long (oligonucleosomes). When run out on a gel, DNA from these cells shows a characteristic 'laddering' pattern (Wyllie et al 1980).

DNA cleaved during apoptotic cell death may be detected in situ by 3' end labelling (Gavrieli et al 1992).

5.1.3 Programmed cell death and luteal regression

Evidence suggesting that apoptosis could be involved in luteal regression comes from a number of sources.

There are morphological similarities between luteolysis and apoptosis, including progressive macrophage infiltration (Lei et al 1991) and nuclear and cytoplasmic condensation (Gillim et al 1969). Such changes were noted in the regressing corpus luteum even before the term 'apoptosis' was coined, (Corner 1956; O'Shea 1977) and are present in steroidogenic and endothelial cells of the gland.

Nucleosomal DNA breakdown was first demonstrated in luteinised granulosa cells in the rat (Zeleznik et al 1989) and has since been demonstrated during spontaneous or induced luteolysis in the cow (Juengel et al 1993), marmoset (Young et al 1996) and human (Shikone et al 1996).

3' end labelling of DNA in apoptotic cells has been demonstrated during luteolysis in women and primates (Young et al 1996; Shikone et al 1996; Funayama et al 1996). The majority of apoptotic cells identified with this method belong to the steroidogenic cell compartment. Cyclical changes in the rate of

apoptosis are thought to play a part in the regulation of other hormone dependant tissues such as endometrium and breast.

5.1.4 Control of programmed cell death

The control of apoptotic rates during luteal maintenance or regression may play a major role in regulating luteal lifespan.

Programmed cell death is controlled by the expression of a number of regulatory genes including c-myc, p53 and APO1/ fas (Osborne and Schwartz 1994). In particular, it has been suggested that an interaction between the proto-oncogene c-myc, and members of the bcl-2 family may be of importance in controlling the rate of apoptosis (Bissonette et al 1992). C-myc expression moves the cell to a position where a choice between proliferation or death must be made. The fate of the cell then depends upon external factors such as the availability of growth factors or internal factors such as the expression of bcl-2 family proto-oncogenes. In the course of our studies c-myc has been demonstrated in the human corpus luteum, although the level of expression remains unchanged throughout the luteal phase (Illingworth et al 1994).

5.1.5 Bcl-2 family of proto-oncogenes

Bcl-2 is one of an expanding family of regulatory proteins and is found in mitochondrial, endoplasmic reticulum and nuclear membranes (Hockenbery et al 1990; Monaghan et al 1992). The bcl-2 gene was first identified from the t(14;18) breakpoint in human follicular lymphoma (Tsujimoto and Croce 1986), and is of particular interest with respect to the rescue of the corpus luteum as it is known to protect against apoptosis (Vaux et al 1988). It has recently been demonstrated that bcl-2 expression may be involved in the selection of ovarian follicles (Tilly 1993) and in the control of the endometrium (Gompel et al 1994).

It is now apparent that an essential component of bcl-2 function is its interaction with bax, a 21kda protein with a degree of homology to bcl-2. Bcl-2 is thought to

form heterodimers with bax, resulting in a loss of its protective effect. Bax itself forms homodimers which induce apoptosis. It appears to be the relative ratios of bcl-2 and bax that will determine the fate of a cell, rather than the absolute levels of either (Oltvai et al 1993; Yin et al 1994). Other bax-like proteins exist which can influence the fate of a cell in different ways (Boise et al 1993 ; Nunez and Clarke 1994).

In the follicle, a decrease in the bcl-2 to bax ratio favours atresia, and similar mechanisms may extend beyond ovulation to govern the fate of the corpus luteum. Indeed, bax expression has recently been shown to be upregulated towards the end of the luteal phase in the cow (Rueda et al 1997), and it is possible that changes in bcl-2 or bax expression at the end of the luteal phase could influence the fate of the gland.

5.1.6 Aims of this chapter

This chapter investigates whether bcl-2 and bax are involved in the regulation of luteal lifespan in women. Immunohistochemistry, protein electrophoresis and the polymerase chain reaction were used to examine bcl-2 and bax expression in corpora lutea retrieved at different stages of the luteal phase as well as after treatment with hCG to mimic luteal rescue in early pregnancy. In addition, the production of bcl-2 protein during induced luteal regression was studied in the marmoset monkey by SDS-PAGE and immunoblotting.

5.2 Materials and Methods

5.2.1 Bcl-2 immunohistochemistry

Sections (5μm) were cut, air-dried at 56^{o}C, dewaxed in xylene then rehydrated in 100%, 95%, and 70% ethanol. Sections were exposed to three 5 minute cycles of microwave irradiation at 700w, in citrate buffer 0.01M, pH 6.0 (Shi et al 1993). Endogenous peroxidase activity was inhibited using 0.3% hydrogen peroxide in methanol for 30 minutes and the sections were preabsorbed for 20

minutes with normal horse serum (Vector, Peterborough, UK). The primary antibody, a monoclonal antibody against a peptide corresponding to amino acids 41-54 of human bcl-2 protein (Dako, High Wycombe, UK) was applied at 1:40 dilution (7.5 mg/mL IgG concentration). For control sections, 7.5 mg/mL of mouse IgG was used in place of primary antibody. After incubation for 20 hours at 4°C, the secondary antibody, biotinylated horse anti-mouse antibody (Vector) was applied for 45 minutes. Colouring was obtained with diaminobenzidine by means of a reaction with avidin covalently coupled to horseradish peroxidase (Vector).

In order to establish which luteal cells localise bcl-2 immunohistochemical colocalisation of bcl-2 and 17-alpha hydroxylase cytochrome P450 was undertaken. 17-alpha hydroxylase is located specifically in the theca lutein cells. (Sasano et al 1989). Eight sections of corpus luteum (2 early, 2 mid, 2 late luteal and 2 rescued) were examined. Sections were dual immunostained for bcl-2 and 17-alpha hydroxylase. Briefly, sections underwent immunohistochemistry for bcl-2 according to the protocol above, using diaminobenzidine as the chromogen. Sections were then blocked with normal swine serum diluted 1:5 in tris buffered saline. Polyclonal 17-alpha hydroxylase cytochrome P450 antibody, raised in a rabbit, (gift of Prof M Waterman, Vanderbilt University, Nashville), was applied at 1:750 dilution, and sections were incubated at 4°C for 20 hours. Biotinylated swine anti-rabbit secondary antibody (Dako) at a dilution of 1:500 was then applied for 30 minutes. Colouring was obtained with nitroblue tetrazium chloride (NBT) by means of a reaction with avidin covalently coupled to alkaline phosphatase (Vector).

Control sections were incubated with normal rabbit serum (Dako) at 1:750 dilution in place of 17-alpha hydroxylase primary antibody.

Staining intensity for bcl-2 was judged by eye by two independent assessors.

5.2.2 SDS-PAGE and immunoblotting

Protein was extracted from eight corpora lutea (2 early, 2 mid, 2 late and 2 rescued). Protein extracted from tonsil was used as a positive control. Tissue was solubilised in 0.1% SDS buffer at 4°C, sonicated and centrifuged for 5 minutes. Supernatant was stored in aliquots at -20°C prior to use. Protein content in the homogenates was determined by Bradford's method using a commercial kit (Bradford 1976; Biorad, Hercules, CA, USA). For polyacrylamide gel electrophoresis (PAGE), 200μg samples were denatured by boiling for five minutes in an equal volume of sample buffer containing 5% β-mercaptoethanol and run on an 11% polyacrylamide gel. Gels were hybridised to a nitrocellulose membrane (Hybond, Amersham International; Buckinghamshire, UK) for 18 hours at 50 volts. Membranes were then were analysed for bcl-2 protein with monoclonal bcl-2 antibody at 1:500 dilution. Proteins were detected with a chemiluminesence technique by means of a sheep antimouse HRP linked antibody (Amersham International).

5.2.3 PCR for bcl-2

Total RNA was extracted from eight corpora lutea (2 early, 2 mid, 2 late and 2 rescued) and one specimen of human tonsil by the method of Chomczynski and Saatchi (1987). The quantity and purity of the nucleic acid preparations were estimated by measuring the optical density of each sample at 260 vs. 280nm. Total RNA was reverse transcribed into first strand cDNA using oligo(deoxythymidine)-primer (Clontech, Palo Alto, CA, USA) and MMLV-reverse transcriptase (Clontech). Oligonucleotide primers were synthesised based on the human bcl-2 cDNA sequence as follows $^{5'}$ ACCTGGATGTTCTGTGCC $^{3'}$, and $^{5'}$ CTCAGACAGAGCCAGTATTGG $^{3'}$. The first strand of cDNA was subjected to 30 cycles of PCR amplification (1 min denaturation at 94°C, 1 min annealing at 70°C and 1 minute extension at 72°C)

and the amplified products were resolved through 1.5% agarose gels. The identity of the 515 base pair amplified product was confirmed by restriction digestion with Hinc II endonuclease.

5.2.4 SDS-PAGE and immunoblotting for bcl-2 during induced luteal regression in the marmoset monkey.

Protein was extracted from eight marmoset ovaries containing corpora lutea. (3 were retrieved from pregnant animals, 2 from non-pregnant control animals, 2 which were retrieved after treatment with PGF2α to induce luteal regression and 1 from an animal which had received GnRH antagonist treatment to induce luteal regression.) In addition, protein was extracted from marmoset thymus for use as a positive control. Tissue was solubilised in 0.1% SDS buffer at 4°C, sonicated and centrifuged for 5 minutes. Supernatant was stored in aliquots at -20°C prior to use. Protein content in the homogenates was determined by Bradford's method using a commercial kit (Bradford 1976; Biorad). For polyacrylamide gel electrophoresis, 200μg samples were denatured by boiling for five minutes in an equal volume of sample buffer containing 5% β-mercaptoethanol and run on an 11% polyacrylamide gel for 6 hours at 180 volts. Gels were hybridised to a nitrocellulose membrane (Amersham International) for 18 hours at 50 volts. Western blots obtained were analysed for bcl-2 protein with polyclonal bcl-2 antibody at 1:500 dilution. Proteins were detected by means of an avidin/biotin linked alkaline phosphatase system (Dako) which reacted with Nitroblue tetrazium chloride (NBT) to give a blue colour.

5.2.5 Bax immunohistochemistry

Sections (5μm) were cut, air-dried at 56°C, dewaxed in xylene then rehydrated in 100%, 95%, and 70% ethanol. Sections were exposed to three 5 minute cycles of microwave irradiation at 700w in distilled water (Krajewski et al

1994a). Sections were preabsorbed for 20 minutes with normal goat serum (SAPU, Carluke ,UK). The primary antibody, a polyclonal antibody directed against a peptide corresponding to amino acids 43-61 of mouse bax protein (Krajewski et al 1994a) was applied at 1:500 dilution. For control sections, normal rabbit serum at a 1:500 dilution was used in place of primary antibody. After incubation for 20 hours at 21°C, the secondary antibody, biotinylated goat anti-rabbit (Dako) was applied for 45 minutes. Colouring was obtained with Vector Red chromogen by means of a reaction with avidin covalently coupled to alkaline phosphatase.

Staining intensity for bax was judged by eye by two independent assessors.

In order to establish the nature of the luteal cells which stained positively for bax immunohistochemistry for 17–alpha hydroxylase cytochrome P450 was undertaken in adjacent sections to those immunostained for bax. Eight sections of corpus luteum (2 early, 2 mid, 2 late luteal and 2 rescued) were examined. Sections were blocked with normal swine serum diluted 1:5 in tris buffered saline. Polyclonal 17-alpha hydroxylase cytochrome P450 antibody, raised in a rabbit, (gift of Prof M Waterman, Vanderbilt University, Nashville, USA), was applied at 1:750 dilution, and sections were incubated at 4°C for 20 hours. Biotinylated swine anti-rabbit secondary antibody (Dako) at a dilution of 1:500 was then applied for 30 minutes. Colouring was obtained with nitroblue tetrazium chloride (NBT) by means of a reaction with avidin covalently coupled to alkaline phosphatase.

Control sections were incubated with nonimmune rabbit serum (Dako) at 1:750 dilution in place of 17–alpha hydroxylase primary antibody.

5.2.6 SDS-PAGE and immunoblotting for bax

Protein was extracted from eight corpora lutea (2 early, 2 mid, 2 late and 2 rescued). Tissue was solubilised in 0.1% SDS buffer at 4°C, sonicated and centrifuged for 5 minutes. Supernatant was stored in aliquots at -20°C prior to

use. Protein content in the homogenates was determined by Bradford's method using a commercial kit (Bradford 1976; Biorad). 200μg protein samples were denatured by boiling for five minutes in an equal volume of sample buffer containing 5% β-mercaptoethanol and run on an 11% polyacrylamide gel. Gels were hybridised to a nitrocellulose membrane for 18 hours at 50 volts. Western blots obtained (Hybond, Amersham International), were analysed for bax protein with polyclonal bax antibody at 1:2000 dilution. Protein bands were visualised using an avidin/biotin linked horseradish peroxidase system (Dako).

5.2.7 PCR for bax

Total RNA was extracted from eight corpora lutea (2 early, 2 mid, 2 late and 2 rescued) by the method of Chomczynski and Saatchi (1987). The quantity and purity of the nucleic acid preparations were estimated by measuring the optical density of each sample at 260 vs. 280nm. Total RNA was reverse transcribed into first strand cDNA using oligo(deoxythymidine)-primer and MMLV-reverse transcriptase (Clontech). Oligonucleotide primers were synthesised based on the human bax cDNA sequence as follows $^{5'}$ TTCTGACGGCAACTTCACTGG $^{3'}$, and $^{5'}$ GCCACAAAGATGGTCACG $^{3'}$. The first strand of cDNA was subjected to 30 cycles of PCR amplification (1 min denaturation at 94°C, 1 min annealing at 53°C and 1 minute extension at 72°C) and the amplified products were resolved through 1.5% agarose gels. The identity of the 223 base pair amplified product was confirmed by restriction digestion with BamH1 endonuclease.

In order to ensure that equivalent quantities of cDNA were used for each reaction, a control PCR reaction was undertaken using primers based on human GAPDH, a housekeeping gene.

5.3 Results

5.3.1 Bcl-2 immunohistochemistry

Photomicrographs of early, mid, late and rescued corpora lutea are shown in figure 5.1 along with control sections obtained with mouse IgG. Positive staining for bcl-2 was found in corpora lutea from all stages of the menstrual cycle including those where maintenance of luteal function with hCG had been performed. Negative control sections were consistently free of staining. Theca lutein cells were identified by double staining for 17-alpha hydroxylase, while granulosa lutein cells were identified on morphological grounds. Examination of double stained sections revealed that all granulosa lutein cells and a majority of theca lutein cells exhibited specific staining for bcl-2.

No difference was observed in the intensity or localisation of immunostaining for bcl-2 between corpora lutea from different stages of the luteal phase. There was no change in the intensity of immunostaining in the hCG rescued corpora lutea.

Bcl-2 localisation was also observed in some of the vessels at the periphery of the parenchymal cells of the corpus luteum. Where such staining was present, it occurred in all endothelial cells of a sectioned vessel. The remaining vessels remained free from staining. The proportion of vessels staining positively for bcl-2 varied from 5% to 15% in individual sections. Variations in this proportion were unrelated to the stage of the luteal phase. There was no change in either the number of cells exhibiting 17-alpha hydroxylase staining or the intensity of staining throughout the luteal phase.

5.3.2 SDS-PAGE and immunoblotting for bcl-2

A single 26kDa band consistent with bcl-2 protein was detected by immunoblotting in all corpora lutea tissue extracts and in tonsil. As can be seen

in figure 5.2, small variations in band intensity were noted but these were unrelated to timing of the sample in the luteal phase.

5.3.3 PCR for bcl-2

A 515 base pair product consistent with bcl-2 was identified after PCR in samples containing cDNA from human tonsil. No such band was present after PCR in samples containing human luteal cDNA. Identity of the product as bcl-2 was confirmed by restriction digestion with Hinc II.

5.3.4 SDS-PAGE and immunoblotting for bcl-2 during induced luteal regression in the marmoset monkey.

A single 26kDa band consistent with bcl-2 was present in the lane containing protein extracted from marmoset thymus. In lanes from marmoset ovary no such bands were present. Marmoset ovary proteins exhibited a band reacting with polyclonal bcl-2 antibody at 31 and 36kDa, with variable numbers of smaller bands at higher molecular weights (figure 5.3).

5.3.5 Bax immunohistochemistry

Immunostaining for bax was observed in all of the sections examined. Staining was identified in granulosa and theca lutein cells. All cells morphologically identified as granulosa cells immunostained for bax. Between 40 and 60% of granulosa lutein cells stained strongly for bax (figure 5.4), with the remainder containing weaker immunostaining. Theca lutein cells also contained bax immunostaining at similar intensity to that of granulosa lutein cells (figure 5.5). Staining intensity was homogenous throughout theca lutein cells. Immunostaining for bax was also identified in 5-15% of blood vessels at the periphery of the gland (figure 5.4). Negative control sections incubated with nonimmune serum in place of primary antibody were consistently free from immunostaining (figures 5.4,5.5). Theca lutein cells were identified by staining

for 17-alpha hydroxylase, while granulosa lutein cells were identified on morphological grounds.

No differences were found in the number of steroidogenic cells staining positively for bax protein, nor the intensity of their immunostaining throughout the luteal phase or in simulated early pregnancy. The number of vessels staining for bax and the intensity of staining did not vary throughout the luteal phase or in simulated early pregnancy.

5.3.6 SDS-PAGE and immunoblotting for bax

A 21 kDa protein, consistent with bax (Oltvai et al 1993), was observed at all stages of the luteal phase in women. Intensity of this band did not vary throughout the luteal phase or in simulated early pregnancy (figure 5.6).

5.3.7 PCR for bax.

A 233 base pair fragment of cDNA was detected at all stages of the luteal phase and in simulated early pregnancy (figure 5.7). The identity of the fragment was confirmed by restriction digestion with BamH1 into two fragments of 152 and 81 base pairs. PCR for GAPDH control confirmed that equivalent quantities of cDNA were used in each reaction.

5.4 Discussion

5.4.1 General Points

This is the first demonstration of the bcl-2 family of proto-oncogenes in the human corpus luteum. Bcl-2 was localised specifically by immunohistochemistry to steroid secreting cells, and the identity of the protein was confirmed by a 26kDa protein band on gel electrophoresis in the human corpus luteum. Bax was similarly localized to the steroidogenic cells of the gland, with identity of the protein detected confirmed by detection of a 21kDa band on protein electrophoresis.

5.4.2 Bcl-2 localisation in steroid secreting cells.

Bcl-2 immunostaining was consistently localised in the cytoplasm of granulosa cells. All cells identifiable as granulosa cells by morphology stained positively for bcl-2. In addition, small clusters of theca lutein cells exhibited bcl-2 staining. Approximately 10% of blood vessels contained bcl-2 immunostaining. No staining was present in supporting cells. The presence of bcl-2 in the steroid secreting cells of the corpus luteum suggests a potential functional role.

Bcl-2 is known to protect against programmed cell death triggered by a wide range of factors (Reed 1994). Some of these factors, for example c-myc, tumour necrosis factor α and reactive oxygen species, have been implicated in luteolysis (Behrman et al 1993). Bcl-2 may prevent death of functional luteal cells that would otherwise occur in response to such stimuli.

5.4.3 Bax localisation in steroid secreting cells

Bax protein was consistently localised in the cytoplasm of granulosa lutein cells, theca lutein cells and a proportion of blood vessels. This pattern of immunostaining is similar to the distribution of bcl-2 in the human corpus luteum, and may indicate that bcl-2 and bax interact to cause changes in apoptotic rates in vivo. Staining intensity varied between granulosa lutein cells within each section, but no morphological differences were detected between granulosa lutein cells which stained strongly or weakly for bax. Upregulation of bax expression of bax prior to apoptosis in specific granulosa lutein cells may result in increased bax immunostaining in particular cells.

5.4.4 Proto-oncogenes in the luteal vasculature

Moderate immunostaining for bcl-2 and bax was also seen in the vascular endothelium of some luteal arterioles and venules at the periphery of the gland, while other vessels remained free from staining. There were no apparent

morphological differences between vessels which exhibited immunostaining and those which did not. These observations suggest that certain luteal vessels may be more or less susceptible to apoptotic cell death depending on relative levels of bcl-2 and bax proteins. Endothelial apoptosis has been observed in the regressing corpus luteum of sheep and may be responsible for reduced blood flow to the gland during luteolysis (O'Shea et al 1977). However, as vascular staining for bcl-2 and bax staining showed little variation throughout the luteal phase, it is unlikely that such a mechanism would be wholly responsible for changes in luteal function at the time points examined.

5.4.5 Absolute and relative production of bcl-2 and bax with changing luteal function.

The process of luteal regression may involve a number of factors. It is possible that functional luteolysis primarily involves uncoupling of the LH receptor, while structural luteolysis involves expression or inactivation of proto-oncogenes involved in apoptosis. A reduction in bcl-2 expression or an increase in bax expression at the end of the cycle may allow luteolysis to proceed. However, as described above, there is no significant change in granulosal, thecal or vascular bcl-2 staining throughout the luteal phase. Corpora lutea which are rescued with hCG to reproduce the hormonal environment of early pregnancy might be expected to have increased production of bcl-2 as part of the mechanism which prevents luteolysis. However, neither the immunohistochemical localisation nor protein electrophoresis revealed any difference to untreated corpora lutea.

Bax protein and mRNA were detected at all stages of luteal function. In contrast to recent observations in bovine corpora lutea, there was no increase in bax expression in the late luteal phase (Rueda et al 1997). This implies that the cell death which occurs during luteolysis in women is not associated with a gross change in the bcl-2/ bax ratio.

5.4.6 Explanations for constant levels of bcl-2 and bax.

Bcl-2 protein may have alternative functions in the human corpus luteum. The mechanism of action of bcl-2 is poorly understood, but its localisation in the membranes of the mitochondria, endoplasmic reticulum and nucleus would be consistent with a role in intracellular transport and regulation. It is possible that bcl-2 production in the human corpus luteum is not linked to the apoptotic process, but has an alternative function within the cell.

Bax may also have alternative functions in the human corpus luteum. The mechanism of action of bax is poorly understood, but as it displays sequence homology to bcl-2, a role in intracellular transport is possible.

Other members of the bcl-2 family such as mcl-1 (Kozopas et al 1993), bak (Farrow et al 1995) and bcl-x (Boise et al 1993) can form heterodimers with bax and bcl-2 as well as acting independently of these factors to promote cell death or survival. The distribution of such factors within the human corpus luteum is not known although bcl-x (Krajewski et al 1994b) and mcl-1 (Krajewski et al 1995) have been detected in murine corpora lutea. Such complex interactions may affect the rate of apoptosis in luteal cells as function of the gland changes.

5.4.7 Bcl-2 production in the marmoset ovary.

Western blotting demonstrated a band consistent with bcl-2 in the marmoset thymus, but protein bands reacting with this antibody in marmoset ovary were of a higher molecular weight. There is no published description in any species of a bcl-2 protein with a molecular weight of 31 or 36kDa. Furthermore, the bcl-2 antibody detected a 26kDa band consistent with bcl-2 in positive marmoset thymus, where a high level of bcl-2 expression was expected, demonstrating that the discrepancy in size is not the result of a species difference in the bcl-2 protein. In the absence of a 26kDa band in marmoset ovary while such a band is present in thymus it seems unlikely that bcl-2 is present in significant levels in the marmoset ovary. The larger bands detected may be the result of a cross

reaction with an unrelated protein, but it is also possible that the bcl-2 antibody used detects a structurally related member of the bcl-2 family in the marmoset ovary.

The detection of no bcl-2 in the marmoset ovary by SDS-PAGE and immunoblotting implies that between primates species differences exist in the expression of bcl-2 family proto-oncogenes in the ovary.

5.4.8 Overview

In summary, bcl-2 and bax are produced by the granulosa, theca and vascular endothelial cells of the human corpus luteum. Production does not appear to change throughout the luteal phase.

Elucidation of the potential roles of proto-oncogenes in the corpus luteum may require further investigations on the wider bcl-2 family of proto-oncogenes once the functions of individual members have been clarified.

Figure 5.1

Photomicrographs of bcl-2 immunostained corpora lutea from throughout the luteal phase. Section (a) is from a rescued corpus luteum and has been stained for bcl-2. Granulosa lutein (G) and theca lutein cell (T) staining are demonstrated. Similarly sections (b), (c), and (d) are stained for bcl-2 and are early, mid and late luteal respectively. Sections (e) and (f) are mid cycle and are serial to (c); (e) is a negative control and (f) is double stained for bcl-2 (brown) and 17 alpha hydroxylase (blue). Sections (g) and (h) are respectively negative control and bcl-2 stained sections showing vascular endothelial staining.

In sections (a) - (f) the bar represents 100μm, and in sections (g) and (h) the bar represents 20.6μm.

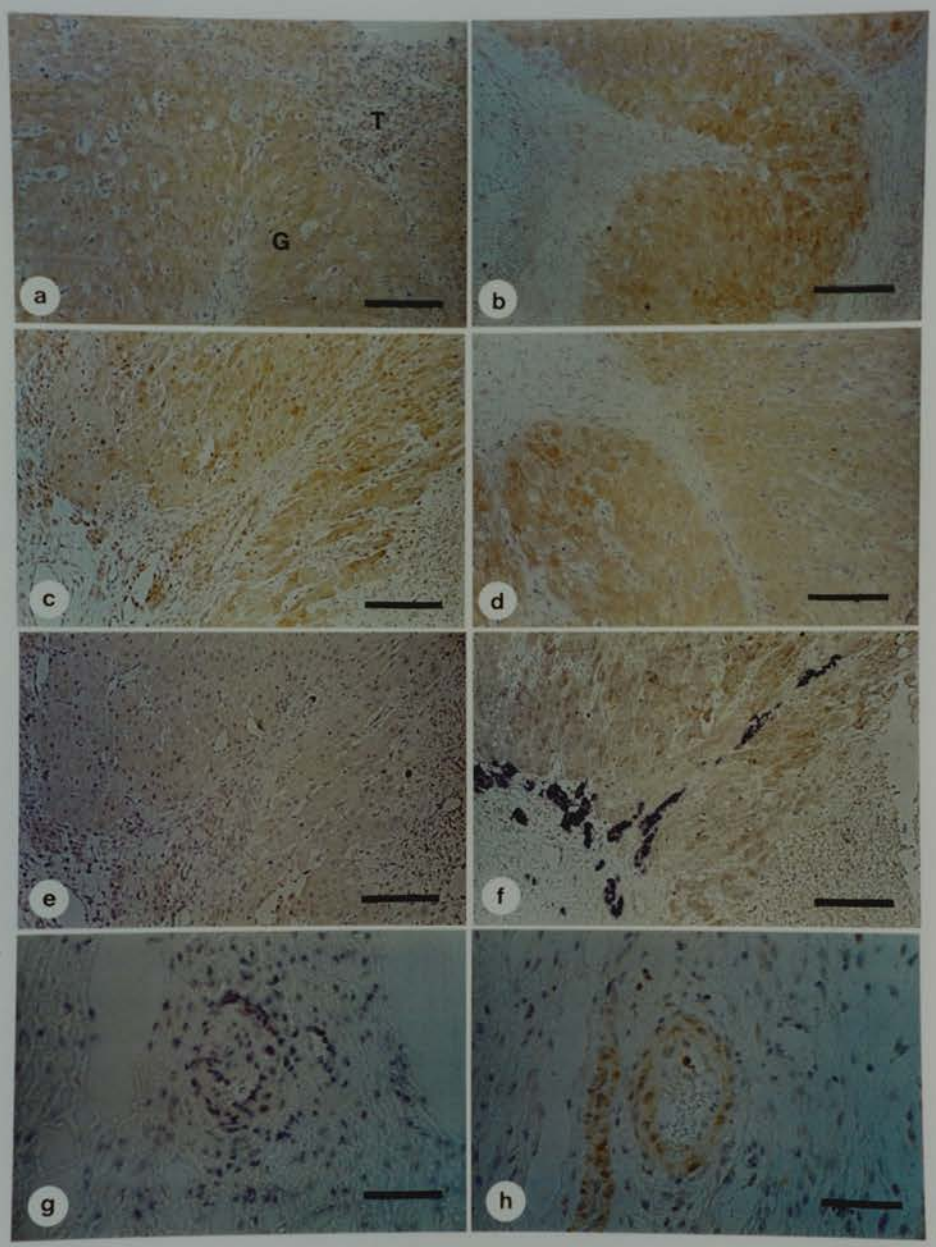

T
G
a
b
c
d
e
f
g
h

Figure 5.2

SDS-PAGE and immunoblotting for bcl-2 throughout the luteal phase. A 26 kDa protein band consistent with bcl-2 is present in early (E), mid, (M), late (L) and rescued (R) corpora lutea and also in tonsil (T), used as a positive control tissue. Molecular weight markers are indicated by numerical values to the left of the figure.

SDS PAGE for bcl-2 in the Human Corpus Luteum

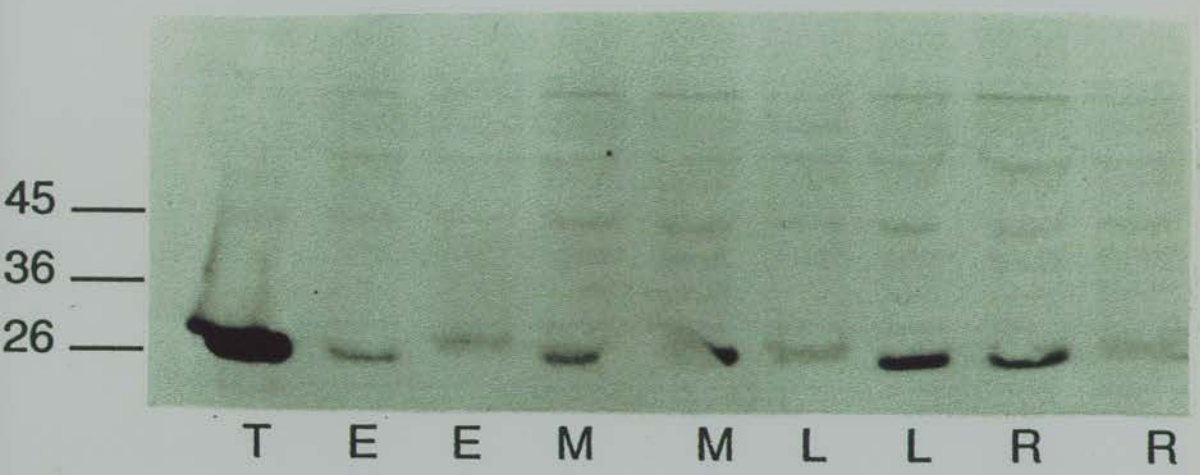


Figure 5.3

SDS-PAGE and imunoblotting for bcl-2 in the marmoset ovary. A 26 kDa band, consistent with bcl-2 is present in human tonsil (T), used as a positive control tissue. Protein samples from non-pregnant, non treated control marmosets (C), pregnant marmosets (P) and animals undergoing induced luteal regression (R) in contrast gave a larger band of approximately 30kDa on immunoblotting.

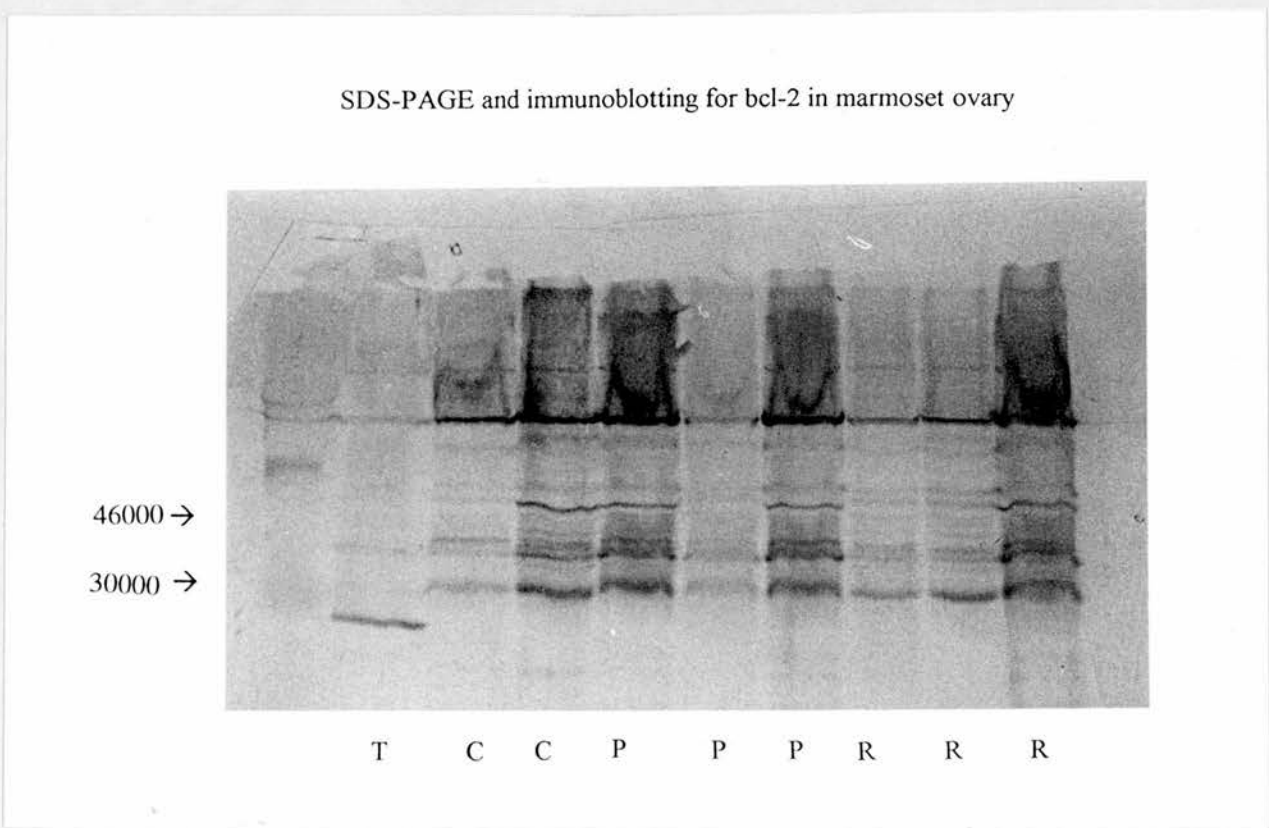


Figure 5.4

High power view of bax immunostaining in early (a), mid (b), late (c) and rescued (d) corpora lutea. Negative control section incubated with non-immune serum in place of primary antibody (e) is free from immunostaining. Luteal blood vessel (f) with positive immunostaining for bax. The bar represents 25μm.

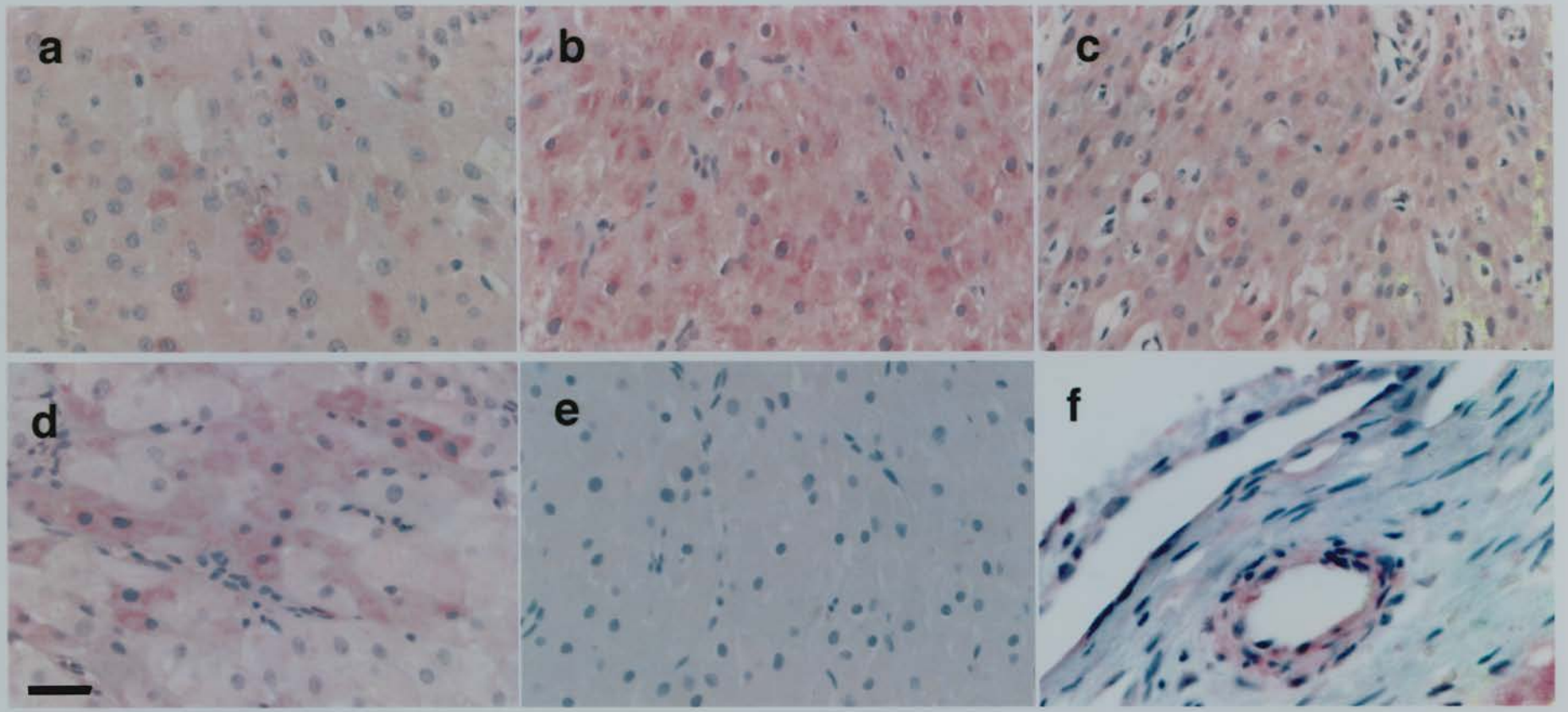

a
b
c
d
e
f

Figure 5.5

Low power view of bax immunostaining in early, mid, late and rescued corpora lutea. Granulosa lutein cells are indicated by the letter G and theca lutein cells by the letter T. In section d the insert shows a serial section immunostained for P450C17. Negative control section incubated with non immune serum in place of primary antibody (e) is free from immunostaining. Tonsil (f) shows immunostaining for bax in a germinal centre (GC). The bar represents 100μm.

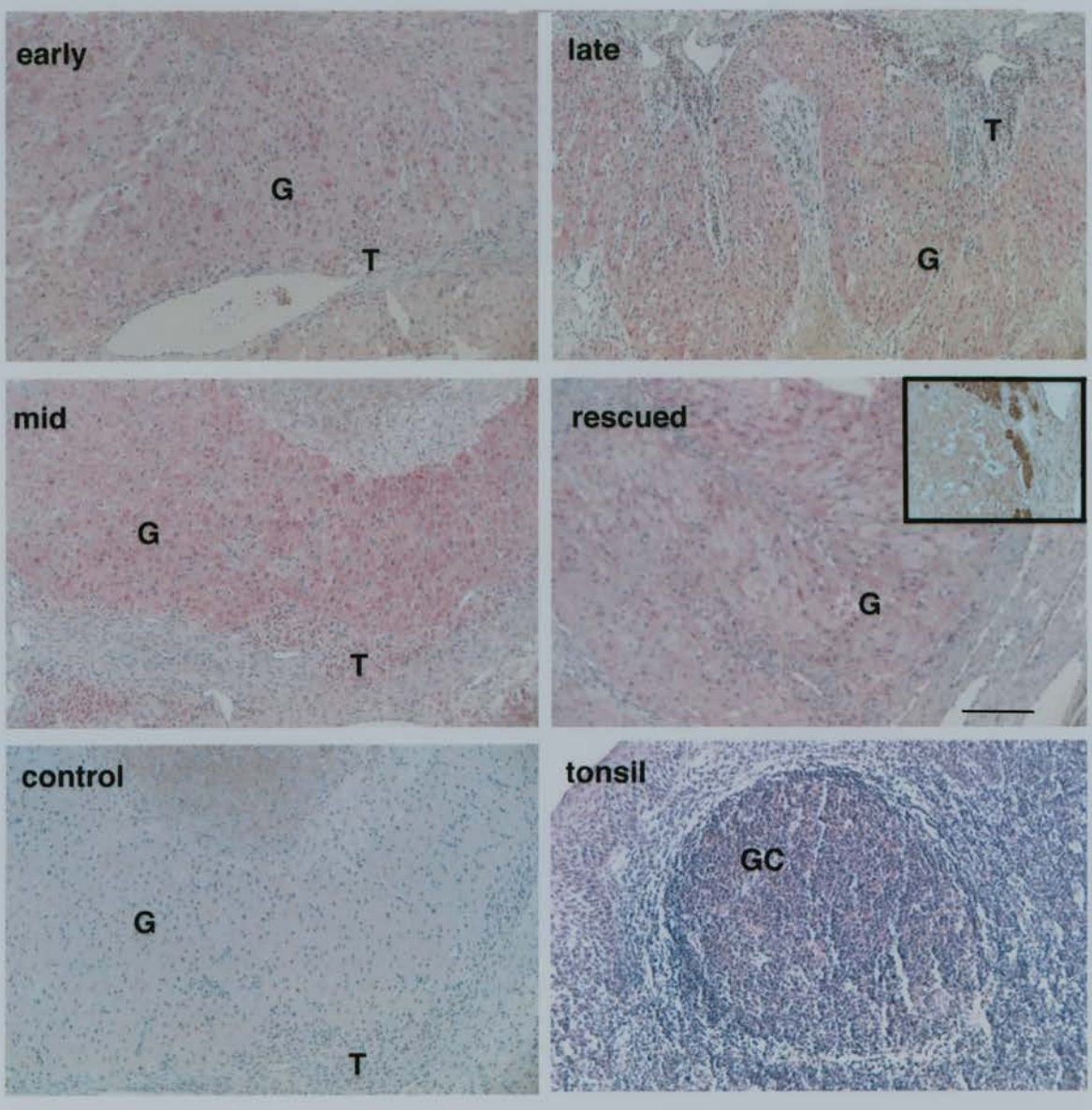

early
G
T
late
T
G
mid
G
T
rescued
G
control
G
T
tonsil
GC

Figure 5.6

SDS-PAGE and immunoblotting for bax throughout the luteal phase. Protein samples are identified as follows: E, early; M, mid; L, late; R, rescued; T, tonsil. A single protein band was detected at 21kDa, consistent with bax, throughout the luteal phase and in simulated early pregnancy.


← 30000
← 21500
← 14300
E E M M L L R R T

Figure 5.7

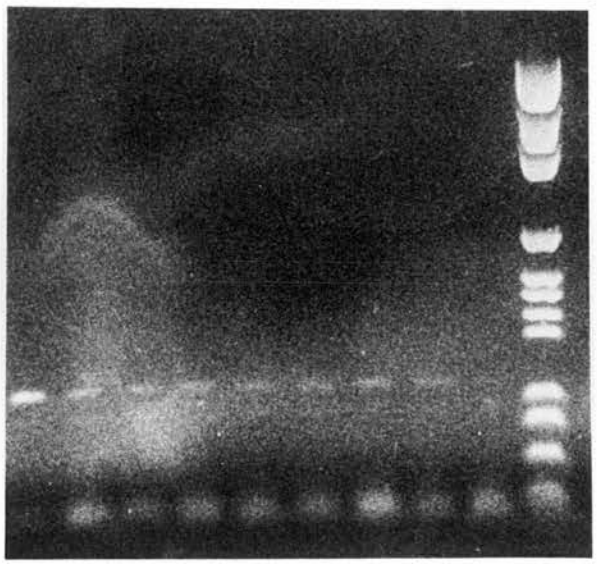
Polymerase chain reaction for bax throughout the luteal phase.

A 233 base pair cDNA amplimer, consistent with bax, was obtained using c DNA templates from the early (E), mid (M), late (L), and rescued (R) stages of the luteal phase.

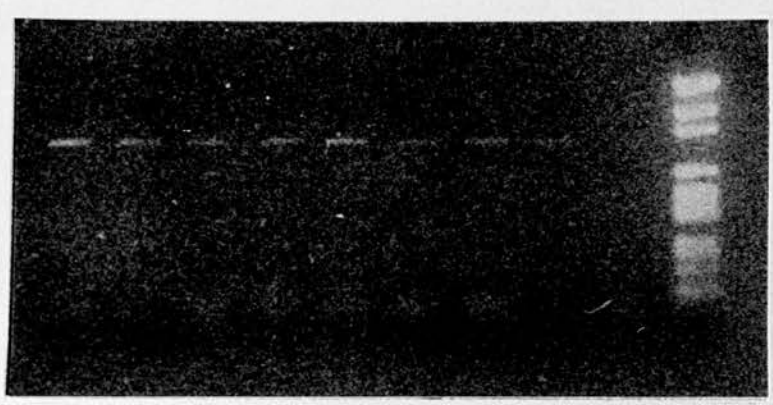
A 956 base pair fragment consistent with GAPDH, a housekeeping gene, is amplified at a constant level from corresponding cDNAs confirming that equivalent quantities of template were used for each reaction.

Negative control PCR mixes were run with sterile water in place of human luteal cDNA template, no amplimer was observed in the negative control lanes (N).

PCR for bax in human corpus luteum



Control PCR for GAPDH in the human corpus luteum



CHAPTER 6

IMMUNE CELLS AND LUTEAL FUNCTION

6.1 Introduction

6.1.1 General points

The immune system may be important for the control of luteal structure and function. In vitro and in vivo studies suggest that immune cells and their secretory products have the potential to influence steroid production, blood vessel formation and the balance between cell proliferation and death in the corpus luteum (reviewed in chapter 1.7).

Although marked species differences exist, significant changes occur in numbers and distribution of immune cells during luteal maintenance and regression in non-primate species (Brannstrom and Norman 1993).

Recent studies by Duncan et al (1998) have examined macrophage distribution during luteal maintenance and regression in women. Macrophage numbers are significantly lower in hCG treated corpora lutea than in untreated tissue from the late luteal phase. It is not known whether numbers or distribution of other immune cell types vary in a similar manner with changing luteal function.

6.1.2 Mediators of changes in cell numbers- MCP-1

Duncan et al (1998) record an attenuation of the increase in macrophage numbers observed during luteal regression after hCG administration. As hCG acts through the common LH receptor on steroidogenic cells it is likely that paracrine mediators are involved. Studies in rat ovary have indicated that MCP-1, a potent macrophage chemoattractant protein, may be implicated (Townson et al 1995; 1996). hCG may act directly or by increasing progesterone production which in turn may down-regulate MCP-1 production (Kelly et al

1997). This would conversely allow secretion of MCP-1 to increase as progesterone production falls during luteal regression.

MCP-1 is localised in steroidogenic cells of rat corpora lutea (Hosang et al 1994), and expression increases during luteal regression but is attenuated in early pregnancy (Townson et al 1995; 1996). It is not known whether MCP-1 is expressed in human corpora lutea or whether secretion follows a similar pattern during luteal maintenance and regression in women.

6.1.3 Aims of chapter

This chapter aims to supplement the work which already details macrophage numbers at different stages of luteal function and in luteal rescue (Duncan et al 1998) by identifying the distribution and numbers of immune cell subsets in women throughout the luteal phase and in simulated early pregnancy. Specific markers examined will comprise: (a) CD45, an antigen common to leukocytes which will detect lymphocytes, monocytes, macrophages and granulocytes (Schwinzer 1989); (b) CD3, a specific marker for T lymphocytes and (c) neutrophil elastase, which is a marker of neutrophilic granulocytes.

This chapter will also explore potential mechanisms underlying the influx of macrophages in the late luteal stage by examining the expression of MCP-1 at different stages of luteal function in women.

6.2 Materials and Methods

6.2.1 Immunohistochemistry for CD45

5μm sections were cut from early (n=6), mid (n=6), late (n=6) and rescued (n=6) corpora lutea. In addition 5μm sections of human tonsil were included in each run to serve as positive control tissue. Sections were dewaxed in xylene and rehydrated in 100%, 95% and 70% ethanol. Antigen retrieval was performed by exposing sections to 2 cycles of microwave irradiation at 700w in citrate buffer 0.01M pH6.0.

Sections were subsequently blocked with normal horse serum diluted 1:5 in tris buffered saline (SAPU, Carluke, UK). Monoclonal anti CD45 antibody, raised against human peripheral blood lymphocytes (Dako, High Wycombe, UK) was applied at a 1:25 dilution (12μg/mL) and sections were incubated overnight at 4°C. Negative control sections were incubated with 12μg/mL of non-immune mouse IgG in place of primary antibody.

After application of biotinylated horse antimouse secondary antibody (Vector, Peterborough, UK) an avidin/biotin alkaline phosphatase detection system was used. This reacted with Vector Red chromogen to produce red immunostaining in CD45 positive cells.

Numbers of CD45 positive cells were counted in four x100 power fields from each section. In all cases the observer was unaware of the nature of the section being examined. Preliminary investigations showed that increasing the number of fields above four did not increase statistical accuracy of the estimations (for early corpora lutea examination of eight fields per section resulted in a mean of 8 positive cells per field, confidence limits 4-12). Differences in the number of cells expressing antigen per high power field were investigated by one-way analysis of variance using stage of the luteal phase as a between subject (corpus luteum) variable with Fischers PLSD test. The numbers of antigen expressing cells in late and rescued corpora lutea were also compared in the same manner.

6.2.2 Immunohistochemistry for neutrophil elastase

Sections were dewaxed and rehydrated as for CD45 immunostaining. Sections underwent proteolytic digestion in 0.1% trypsin pH7.4 at 37°C for 45 minutes and were then blocked with horse serum as above.

Monoclonal antibody directed against human neutrophil elastase was applied at 1:50 dilution and sections were incubated at 37°C for 1 hour. Negative control

sections were incubated with an equivalent concentration of mouse IgG in place of primary antibody.

As for CD45 staining, an alkaline phosphatase-linked avidin-biotin detection system was used. Vector Red gave red immunostaining in positive cells.

Numbers of neutrophils were counted in 4 low power (x40) fields in each section. Examination of more than 4 low power fields was precluded by the size of the luteal biopsies.

6.2.3 Immunohistochemistry for T cells

5μm sections of corpus luteum and tonsil were dewaxed and rehydrated as described above. Sections were blocked in 3% hydrogen peroxide in distilled water for 5 minutes, then digested in 0.1% trypsin pH7.4 for 30 minutes at 37°C. A commercial antibody was applied for I hour at 37°C. The commercial antibody complex used consisted of rabbit anti-T cell CD3 and horse radish peroxidase coupled to an inert polymer backbone and was supplied ready diluted (EPOS Anti-human T cell, Dako). Negative control sections were incubated with EPOS negative control solution. Positive cells were visualised by application of diaminobenzidine chromogen, giving brown immunostaining in positive cells.

6.2.4 SDS-PAGE and immunoblotting for MCP-1

Protein was extracted from eight corpora lutea (2 early, 2 mid, 2 late and 2 rescued). Tissue was solubilised in 0.1% SDS buffer at 4°C, sonicated and centrifuged for 5 minutes. Supernatant was stored in aliquots at -20°C prior to use. Protein content in the homogenates was determined by Bradford's method using a commercial kit (Bradford 1976; Biorad, Hercules, CA, USA). For polyacrylamide gel electrophoresis, 200μg samples were denatured by boiling for five minutes in an equal volume of sample buffer containing 5% β-mercaptoethanol and run alongside molecular weight markers (Rainbow

Markers, Amersham International, Buckinghamshire, UK) on an 10% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane for 18 hours at 50 volts. Western blots obtained (Hybond, Amersham), were analysed for MCP-1 protein with polyclonal MCP-1 antibody raised in rabbit (gift of Dr Rodney Kelly, MRC Reproductive Biology Unit, Edinburgh, UK) at 1:250 dilution. Proteins were detected with an avidin/ biotin linked alkaline phosphatase system which reacted with Vector black chromogen to give brown-black bands.

6.2.5 PCR and purification of MCP-1 fragment

Total RNA was extracted from eight corpora lutea (2 early, 2 mid, 2 late and 2 rescued) by the method of Chomczynski and Saatchi (1987). The quantity and purity of the nucleic acid preparations were estimated by measuring the optical density of each sample at 260 vs. 280nm. Total RNA was reverse transcribed into first strand cDNA using oligo(deoxythymidine)-primer and MMLV-reverse transcriptase (Clontech, Palo Alto, CA, USA). Oligonucleotide primers were synthesised based on the human MCP-1 cDNA sequence (Yoshimura et al 1989) as follows $^{5'}$ AATCAATGCCCCAGTCACCTGC $^{3'}$, and $^{5'}$ CGGAGTTTGGGTTTGCTTGTCC $^{3'}$. The first strand of cDNA was subjected to 30 cycles of PCR amplification (1 min denaturation at 94°C, 1 min annealing at 45°C and 1 minute extension at 72°C) with a final 10 minute extension at 72°C. The amplified products were resolved a 1.5% agarose gels.

In addition to a 210bp band consistent with MCP-1 an unidentified band of approximately 600bp was identified on the gel. In order to proceed to subcloning of the MCP-1 fragment, the appropriate band was purified. Gel bands were visualised under UV light. A sterile 16 gauge hypodermic needle with attached 2 mL syringe was inserted into the appropriate MCP-1 band and the plunger was pushed up and down several times in order to draw small quantities of the gel with appropriate cDNA band into the needle. In order to use

the small amount of cDNA withdrawn as a template for PCR, the needle and syringe were then introduced into a 0.5 mL PCR tube containing 50μl of a PCR mastermix containing dNTPs, MCP-1 primers and taq polymerase. This solution was aspirated up and down with the syringe and needle a number of times. This was repeated for MCP-1 cDNA bands from each stage of the luteal phase which were visible on the original gel.

The mixture was then processed for PCR as above. MCP-1 cDNA selected in this way was resolved on a 1.5% gel.

6.2.6 Subcloning

The 210bp fragment obtained as above was ligated into pCRII vector and transformed into competent E-coli cells using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). Plasmid was extracted with a commercial kit (Wizard Prep, Promega, Madison, WI, USA).

In order to check that extracted plasmid contained the insert of interest, restriction digestion with EcoR1 was carried out. This endonuclease restricts on either side of the insert in pCRII.

Plasmids underwent sequencing by Mr J Gaughan (MRC Reproductive Biology Unit) .

6.3 Results.

6.3.1 Immunohistochemistry for CD45

CD45 positive cells were identified in tonsil and in all sections of human corpora lutea examined. Cells with CD45 immunostaining were present between both theca and granulosa lutein cells, in the stromal tissue at the periphery of the gland and in small luteal blood vessels. CD45 positive cells were most numerous at the junction of the theca and granulosa lutein cells, and in the area between the granulosa lutein cells and the central cavity. CD45 positive cells

were also present within the fibrin mass at the centre of the corpus luteum (figure 6.1).

Numbers of CD45 positive cells varied significantly throughout the luteal phase. Numbers per low power field were low in the early luteal phase (9, confidence limits 5-13) rising significantly in the mid luteal phase (38, confidence limits 30-46, $p<0.05$) and rising further still to a peak of 61 in the late luteal phase (confidence limits 44-78). Numbers of CD45 positive luteal cells fell significantly following hCG treatment to 22 (confidence limits 15-29, $p<0.05$) (figure 6.2).

6.3.2 Immunohistochemistry for neutrophil elastase

Cells staining positively for neutrophil elastase were present in human tonsil and in all sections of human corpora lutea examined. Neutrophils, although present , were scarce, and were localised in the granulosa cell layer, the theca cell layer and in the stroma surrounding the gland. Neutrophils were most commonly observed as small clusters, often within small blood vessels in each of the layers described (figure 6.3).

Due to large variations within each of the early, mid, late and rescued luteal groups, numbers of neutrophils did not change significantly throughout the luteal phase. However a trend in neutrophil numbers was observed, with higher numbers observed in the early (33, confidence limits 11-55) and late (37, confidence limits 18-56) corpora lutea, and lower numbers observed in the mid (6, confidence limits 3-9) and rescued corpora lutea (4, confidence limits 1-7). The fall in neutrophil numbers following hCG treatment did not reach statistical significance ($p=0.31$) (figure 6.4).

6.3.3 Immunohistochemistry for CD3

Immunostaining for CD3 was observed in tonsil, used as a positive control tissue. CD3 positive cells were extremely sparse in the human corpus luteum (less than 5 positive cells per section) and were only observed in 50% of

sections. When present, T cells were most commonly located in larger blood vessels or the stroma surrounding the gland. T cells were not observed in granulosa or theca lutein cell layers (figure 6.5). Numbers of T cells did not vary significantly throughout the luteal phase or in simulated early pregnancy.

6.3.4 SDS-PAGE and immunoblotting for MCP-1

Immunoblotting for MCP-1 revealed 3 faint bands at 14 kDa, 18 kDa and 32 kDa. The smallest of these bands is consistent with published reports of MCP-1 in other tissues (Yoshimura et al 1989), while the larger bands may correspond to a glycosylated form of the cytokine or to MCP-1 dimer formation.

The 14 and 18 kDa bands were present throughout the luteal phase and during simulated early pregnancy (figure 6.6).

6.3.5 PCR and purification of MCP-1 fragment

Initial PCR for MCP-1 resulted in the amplification of 2 fragments of cDNA, a smaller 210bp fragment, corresponding to MCP-1, and a larger 600bp fragment. MCP-1 and the larger unidentified fragment were detected in corpora lutea from all stages of the luteal phase (figure 6.7).

After inoculation of a new PCR mix and subsequent amplification, a single 210bp band was obtained in all four reactions (figure 6.7).

6.3.6 Subcloning and sequencing of MCP-1

Restriction digestion with EcoR1 showed that a 210bp fragment had been successfully cloned into the pCRII vector. Sequencing revealed that the plasmid insert showed 99% homology to human MCP-1 invert (Yoshimura et al 1989).

6.4 Discussion

6.4.1 General points

This is the first study to demonstrate the presence of MCP-1 in the human corpus luteum. In addition, this work represents the first description of luteal leukocyte, neutrophil and T cell distribution in simulated early pregnancy in women.

6.4.2 Changes in leukocyte numbers throughout the luteal phase

In agreement with previous studies, number of white blood cells was shown to increase during functional luteal regression (Brannstrom et al 1994a) This investigation has demonstrated that this influx of leukocytes is prevented by hCG treatment. With no significant change in numbers of neutrophils or T lymphocytes at different stages of luteal function it is likely that the changes in white cell numbers are largely due to variations in macrophage numbers.

Although changes in neutrophil numbers throughout the luteal phase did not reach significance there was a trend for greater numbers of these cells in the early and late luteal phases. In other species, an influx of neutrophils has been noted at the time of ovulation (Gerdes et al 1992; Brannstrom et al 1993), and it has been suggested that the ability of these cells to break down the extracellular matrix may contribute to tissue remodelling associated with formation of the corpus luteum (Behrman et al 1993).

Neutrophils have also been implicated in luteal regression in the rat. When incubated with luteinised granulosa cells, neutrophils were associated with a decrease in progesterone secretion (Peperell et al 1992). Neutrophils are capable of free radical production, which has been linked to their luteolytic action in vitro (Behrman and Preston 1989; Brannstrom and Norman 1993). The ability of neutrophils to breakdown the extracellular matrix is likely to be of importance in structural luteal regression, but may also be important for angiogenesis throughout the lifespan of the gland.

T cells were not abundant in the human corpus luteum at any stage of function. Lymphocytes secrete cytokines which have been demonstrated to posses

luteotropic properties in vitro (Emi et al 1991). However no increase in T cell abundance was observed after treatment with hCG to simulate early pregnancy.

6.4.3 Increased leukocyte numbers in luteolysis- potential mechanisms

The increase in numbers of leukocytes during luteal regression may be due to recruitment of white cells from the blood stream and subsequent migration into the gland under the influence of locally produced chemotactic factors. Alternatively macrophages already present in the corpus luteum may proliferate shortly before or during luteolysis. A cohort of proliferating non-endothelial, non steroidogenic cells have been identified in the human corpus luteum (Rodger et al 1997). It is possible that proliferating immune cells contribute to this subset of dividing cells. However, the proliferating non-endothelial non-steroidogenic cells were morphologically similar to steroidogenic cells and shared none of the morphological characteristics of macrophages or neutrophils, the two most prevalent types of human luteal white cells.

Recruitment of leukocytes from the bloodstream involves attachment to the vascular wall, passage between endothelial cells and subsequent migration into the gland). Each of these processes could be observed in CD45 positive cells in the corpus luteum (figure 6.8). These processes may be facilitated by cytokines such as IL-1 and TNFα secreted from immune or steroidogenic cell compartments.

6.4.4 MCP-1 and changing luteal function

MCP-1 was identified in the human corpus luteum by PCR and subcloning, and SDS-PAGE and immunoblotting. MCP-1 was detected at all stages of the luteal phase with no stage dependent changes in MCP-1 levels observed. This finding contrasts with data obtained from the rat corpus luteum (Townson et al 1995; 1996) which suggest that levels of MCP-1 increase during luteal regression.

There are considerable species differences in the distribution and production of other cytokines in the corpus luteum (Brannstrom and Norman 1993), and MCP-1 may have a varying involvement in the control of luteal function in different species.

In the human other local mediators may be involved in macrophage recruitment during luteal regression. Constant levels of MCP-1 in the gland may maintain the baseline numbers of macrophages which may be essential for the function of the gland throughout the luteal phase. MCP-1 also has angiogenic properties (Nakashima et al 1995; Ito et al 1997) and may be involved in the high level of angiogenesis which is typical of the corpus luteum.

6.4.5 Other steroidogenic cell cytokines

Steroidogenic luteal cells may also secrete factors which are chemotactic for other subgroups of immune cells. Interleukin-8 has neutrophil chemoattractant and activating properties in vitro and in vivo (Baggiolini et al 1989) and has recently been identified in human luteinised granulosa cells (Arici et al 1996). Production of this cytokine is negatively regulated by progesterone in a similar way to MCP-1 (Arici et al 1996), but it is unclear whether production of this cytokine controls the ingress of neutrophils in the corpus luteum. IL-8 also has angiogenic properties (Koch et al 1992) and may be associated with control of the luteal vasculature.

6.4.6 The immune system may have a broad role in the control of luteal function

As well as a role in structural luteal regression, leukocytes may have an integral role in wider aspects of luteal function (Alila and Hansel 1984). Cells of the immune system may have luteotropic as well as luteolytic effects (Brannstrom and Norman 1993), and secretory products of white blood cells

may alter rates of proliferation and apoptosis in the corpus luteum as well as influencing luteal angiogenesis.

Where leukocytes are involved in luteal regression, this may be by means of phagocytosis, free radical production or extracellular matrix remodelling.

6.4.7 Overview

This chapter has highlighted the interactions between cells of the immune, endothelial and steroidogenic cell compartments in luteal function. Increased numbers of white blood cells may be involved in luteal regression, but lower numbers of immune cells may be required for optimal luteal function earlier in the lifespan of the gland.

Figure 6.1

Immunostaining for CD45 (common leukocyte antigen in the human corpus luteum). Leukocytes are present in early (a), mid (b), late (c) and rescued (d) corpora lutea. The insert shows a negative control section which was incubated with non-immune mouse IgG in place of primary antibody and is free from immunostaining. The bar represents 200μm

c
a
d
b

Figure 6.2

Graph illustrating numbers of CD45 positive leukocytes per x 100 field throughout the luteal phase and in simulated early pregnancy. Numbers of leukocytes increase significantly in the late luteal phase. Treatment with hCG is associated with a significant reduction in leukocyte numbers.

Leukocyte numbers throughout the luteal phase

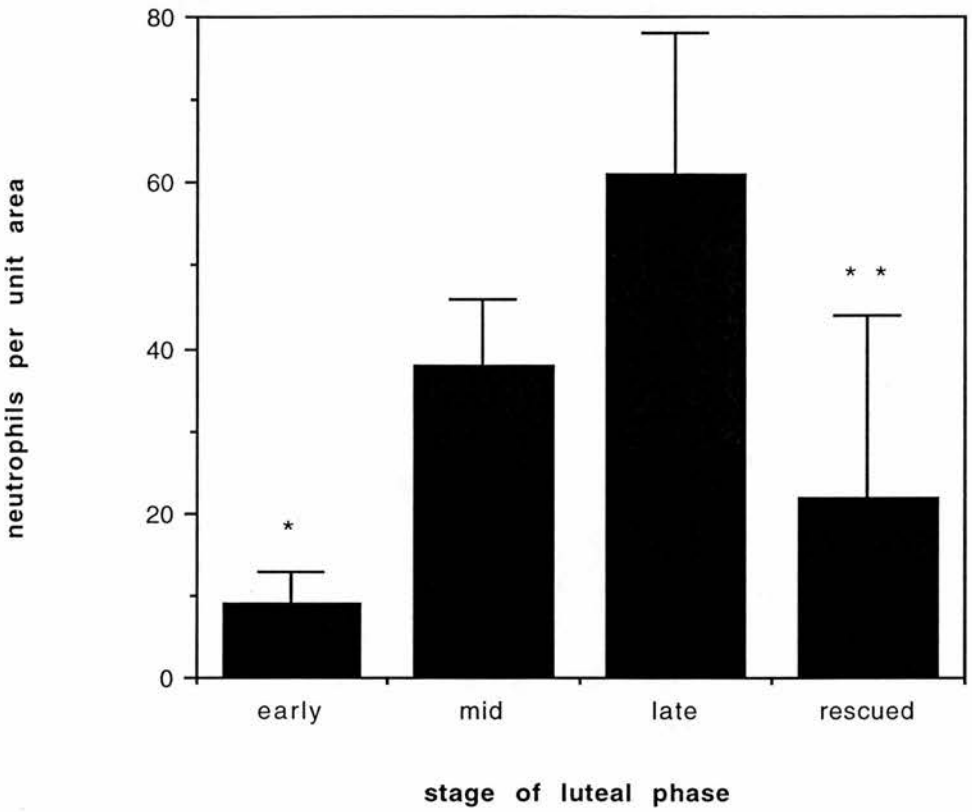


Figure 6.3

Immunostaining for neutrophil elastase in the human corpus luteum and tonsil. Low power view of immunostaining for neutrophil elastase in early corpus luteum (a) with corresponding negative control (b). High power view of a peripheral vessel in the early corpus luteum immunostained for neutrophil elastase (c) with corresponding negative control (d). Negative controls were incubated with mouse IgG in place of primary antibody. Immunostaining for neutrophil elastase in human tonsil, used as a positive control tissue, is shown in (e). In each case the magnification bar represents 50μm.

a
b
c
d
e

Figure 6. 4

Graph illustrating numbers of neutrophils per low power (x40) field throughout the luteal phase and in simulated early pregnancy. There are no significant differences in neutrophil numbers at different stages of luteal function.

Neutrophil numbers throughout the luteal phase

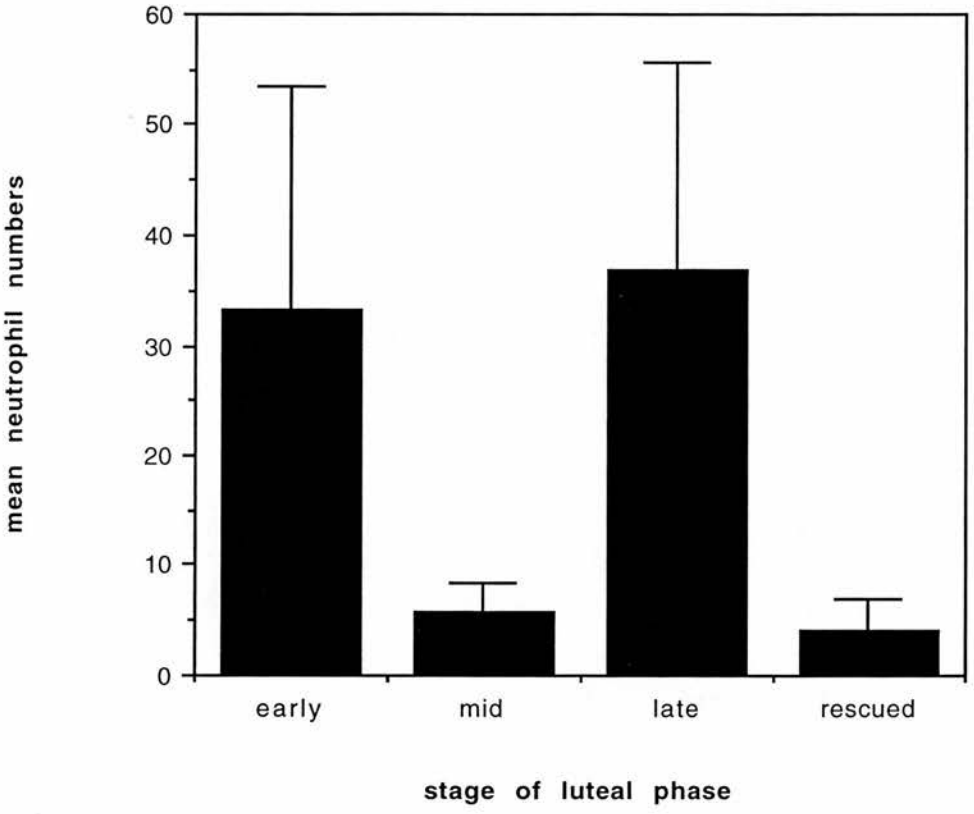


Figure 6.5

Immunostaining for CD3 (T cells) in the human corpus luteum and tonsil. Immunostaining is abundant in tonsil, used as a positive control tissue. T cells in a section of late corpus luteum are shown in (b) (arrow). Lack of T cell staining in the granulosa cell layer of a rescued corpus luteum is demonstrated in (c). In each case the magnification bar represents 50μm.

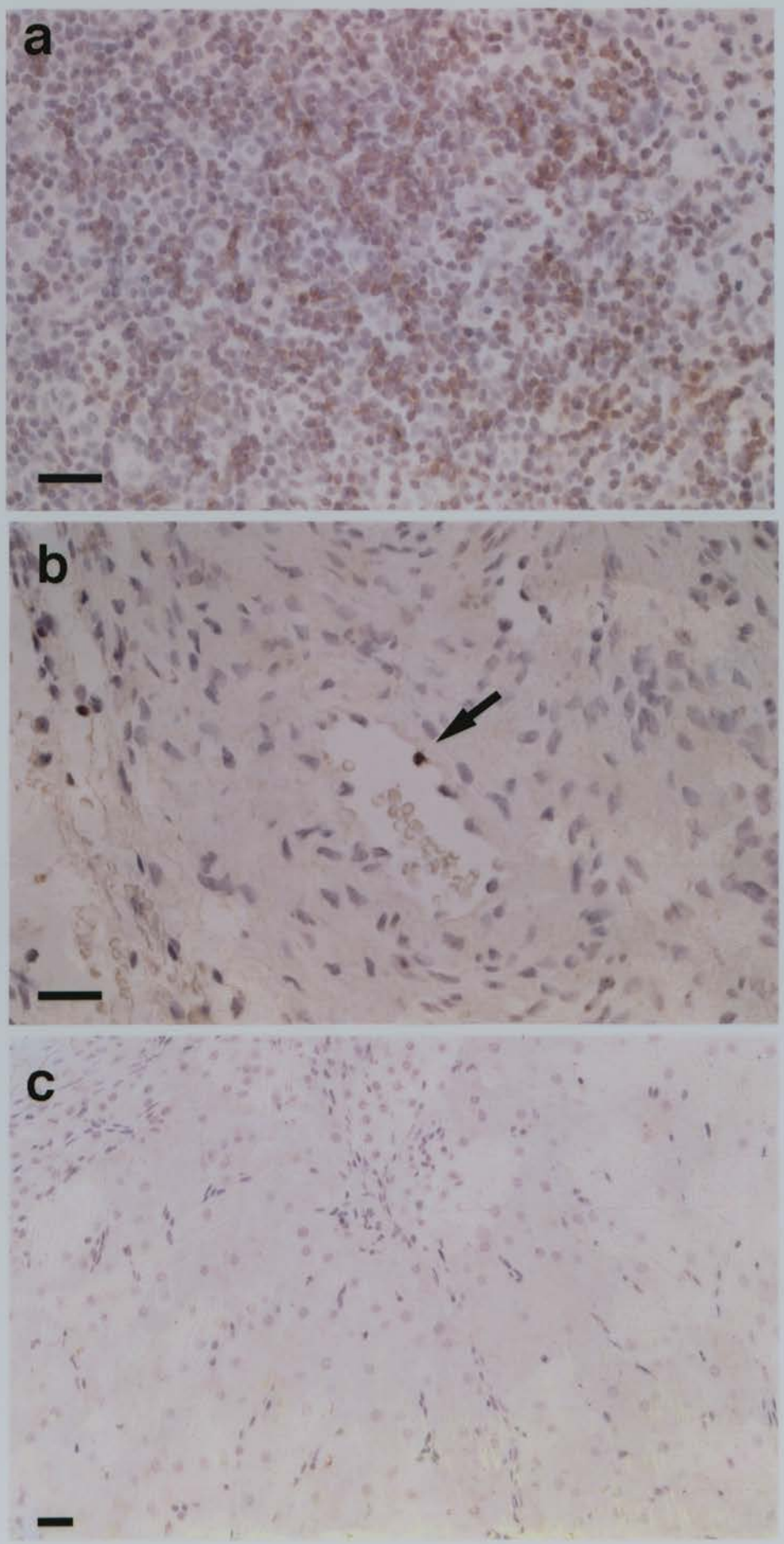

a
b
c

Figure 6.6

SDS-PAGE blotting for MCP-1 in the human corpus luteum throughout the luteal phase. Protein samples are delineated as early, mid, late and rescued. Protein bands, consistent with MCP-1, are present at 14 and 18kDa.

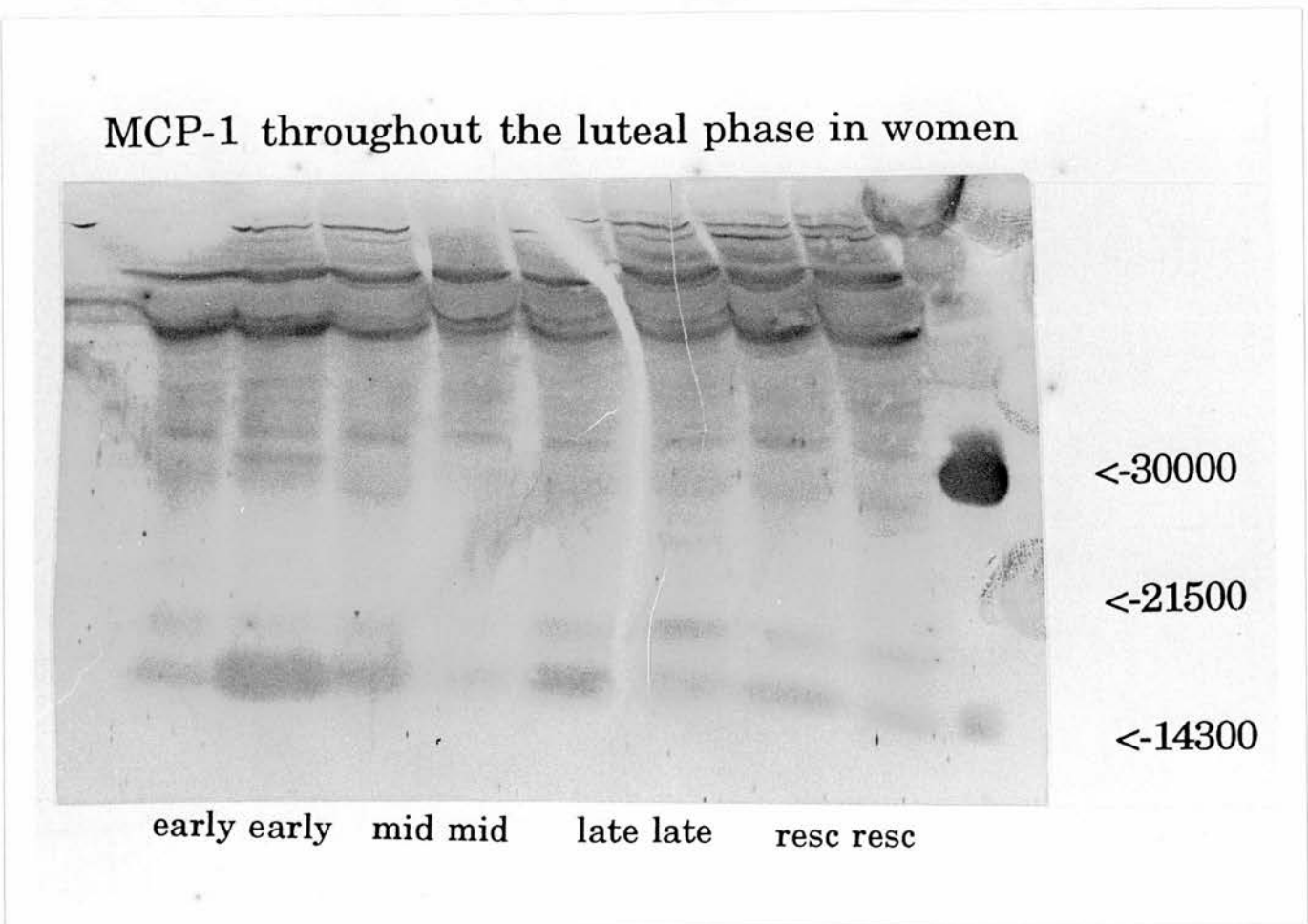
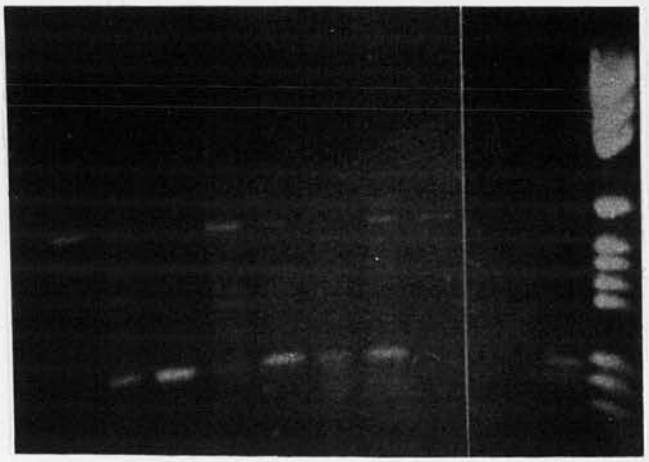


Figure 6.7

(a) Initial PCR for MCP-1 at different stages of luteal function. A 210 base pair band consistent with MCP-1 is observed at all stages of luteal function but in several samples a larger unidentified 600 base pair fragment is visible.

(b) Purification of MCP-1 band from gel. After inoculation of a PCR mix with the appropriate band, a single cDNA band of 210 base pair band consistent with MCP-1 is amplified throughout the luteal phase.

PCR for MCP-1 in human corpus luteum



PCR of purified MCP-1 fragment

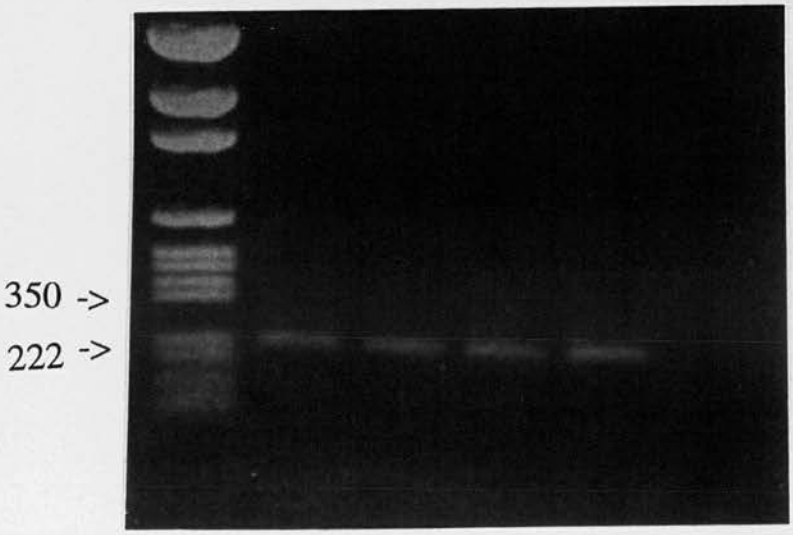
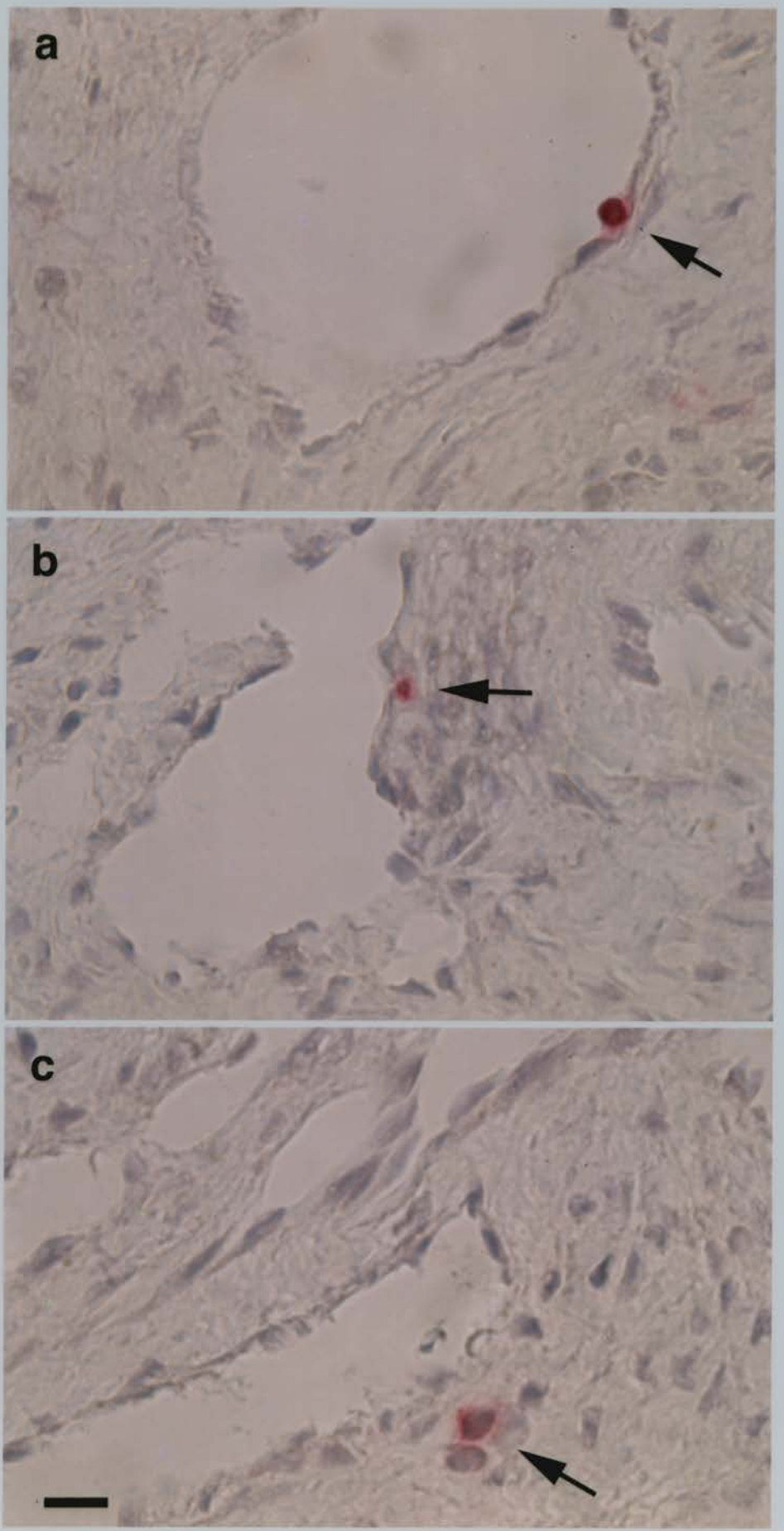


Figure 6.8

Immunostaining for CD45 illustrates recruitment of leukocytes from the bloodstream into the corpus luteum. The leukocyte first attaches to the wall of the blood vessel (a) then passes between the endothelial cells (b) to enter the luteal parenchyma (c). All sections are from the same early corpus luteum, and in each case the magnification bar represents 25μm.


a
b
c

CHAPTER 7

DISCUSSION: SIGNIFICANCE OF NON-STEROIDOGENIC CELLS IN LUTEAL MAINTENANCE AND REGRESSION

7.1 Summary of results

This thesis has aimed to examine the relative roles of different cellular mechanisms in controlling luteal lifespan. Endothelial, immune and steroidogenic cell pathways have been examined throughout the luteal phase. In addition, rates of cell growth and expression of apoptotic proto-oncogenes have been studied. The work described in this thesis has led to speculation that interactions between cells of more than one lineage may regulate luteal function.

The results of these investigations can be summarised as follows:-

1. Cell proliferation is maximal during formation of the corpus luteum, but changing proliferation rates are not associated with luteal rescue in early pregnancy or with luteal regression at the end of a cycle. Endothelial cells represent the main proliferating cell compartment in the human corpus luteum, and steroidogenic cells rarely proliferate.
2. VEGF and bFGF are produced by steroidogenic and endothelial cells respectively throughout the lifespan of the corpus luteum and are produced at constant levels during changes in luteal function. These factors may play a major role in supporting angiogenesis in the human corpus luteum.
3. While apoptosis has been described in the corpus luteum and may be involved in luteal regression, expression of bcl-2 and bax, proto-oncogenes which control apoptotic rates in ovarian follicles and other tissues, do not

change throughout the lifespan of the corpus luteum, in simulated early pregnancy or luteal regression.

4. The largest compartment of immune cells in the corpus luteum is macrophages, but neutrophils and T cells are also present. Total leukocyte numbers are highest during luteolysis and it has been proposed that such cells may be involved in the structural and functional regression of the gland. The human corpus luteum produces MCP-1 which is chemotactic for macrophages. MCP-1 is produced throughout the luteal phase in women.

7.2 The experimental model used : strengths and weaknesses

7.2.1 Description of experimental model used

The investigations described in this study were performed with tissue from an in vivo model of human luteal function. In contrast, much of current understanding of human luteal physiology is based on investigations utilising in vitro luteal systems, in particular luteinised granulosa cells.

The experimental model developed for this thesis involved retrieval and characterisation of luteal biopsies from women undergoing hysterectomy for benign gynaecological disorders. Luteal tissue was accurately dated by clinical and endocrinological parameters. To investigate luteal physiology during simulated early pregnancy a cohort of women received treatment with an incremental regime of hCG which has previously been shown to mimic the endocrine effects of early pregnancy (Illingworth et al 1990).

7.2.2 Strengths of model

This in vivo-model of luteal formation, regression and maintenance confers a number of advantages over in vitro methodology.

Although in vitro studies have yielded data describing steroidogenic cell function under varying conditions, these systems may not accurately represent the physiological processes which occur throughout the luteal lifespan. In such

in vitro systems granulosa cells retrieved from ovarian follicles luteinise in vitro. They are thus isolated from the mechanical and secretory influences of endothelial and immune cells with which they would be in close contact in vivo. Moreover, nutrients and tropic factors are present at regulated levels in culture media of in vitro systems. The physiology of luteal tissue in vivo is however likely to be influenced by variable local factors such as vascular supply and permeability, secretion of growth factors and production of non-secreted factors such as free radicals.

The in vivo model used in this thesis confers a number of additional advantages over in vitro systems.

This model used human luteal tissue. Marked species differences in luteal structure and function occur across domestic species and primates. Examination of human luteal tissue is therefore important to clarify the distinct processes which may control luteal lifespan in our species.

The system of tissue collection utilised in this study ensured that luteal tissue was retrieved prior to ligation of the ovarian vasculature and that tissue was fixed or frozen immediately on retrieval from the patient. This contrasts with other investigators who have examined corpora lutea from routine pathology specimens. Such routine specimens will have been devoid of vascular support for 30 to 60 minutes prior to fixation or freezing. This may lead to increased expression of hypoxia-sensitive gene products and is a possible source of artefact.

This collection system utilised in this thesis formed a luteal tissue bank containing over 60 luteal biopsies from differing stages of luteal function. This permitted examination of adequate numbers of identically fixed and processed and frozen corpora lutea at each stage of the luteal phase.

The experimental model of human luteal function used in this thesis allowed the examination of luteal physiology in intact tissue at a wide range of time points throughout the luteal phase. As individual cell types were not separated, the

importance of steroidogenic and non-steroidogenic cell types and their interactions at times of changing luteal function could be evaluated. Limited manipulation of the system was possible, and administration of hCG to prolong luteal function allowed comparison of luteal maintenance and regression.

7.2.3 Limitations of model

Although this in vivo model allowed examination of the physiology of the corpus luteum as a whole throughout its lifespan, results obtained from these investigations must be considered with reference to the limitations of the experimental model used.

The experimental model utilised tissue retrieved from donors of older reproductive age (median age 40 years). However all luteal donors demonstrated midcycle urinary LH peaks and all subjects had ovulatory progesterone concentrations apart from the late luteal group where the low concentration would be anticipated. In addition Klein et al (1996) has shown that while older women have marked differences in early phase FSH and inhibin B concentrations compared with younger women, there is no difference in the function of either the dominant follicle or the corpus luteum.

It is therefore unlikely that the older reproductive age of the luteal donors would affect the appropriateness of the tissue studied.

Early pregnancy was simulated by an increasing regime of hCG administration in a cohort of luteal donors. hCG stimulation was continued for 6 to 8 days prior to luteal retrieval. This regime has previously been shown to closely mimic the endocrine effects of early pregnancy in terms of serum levels of progesterone and inhibin, but the regime may have important limitations. The short duration of hCG stimulation may give rise to an initial increase in endocrine output of the corpus luteum without allowing time for more complex functional and structural events associated with maternal recognition of pregnancy to occur. Similarly, retrieval of late luteal biopsies was carried out on days 10-14 after ovulation,

and no luteal biopsies were obtained after menstruation had commenced. Initial attempts to remove corpora lutea in the follicular phase of the next ovarian cycle were unsuccessful due to the friable nature of the structurally regressing gland.

It is possible that levels of endothelial cell proliferation and angiogenic growth factor production observed during luteal maintenance and regression in our model undergo changes at later time points during luteal maintenance and regression than examined using this model. Similarly, changing ratios of the apoptotic proto-oncogenes bcl-2 and bax may not be observed at the early time points examined.

Alternatively, it is possible that currently unknown extraovarian factors are important for luteal maintenance. If this was the case then hCG stimulation alone may not give an accurate representation of the changes occurring during physiological luteal rescue. However no such factors have been identified, and as mentioned previously, hCG administration is associated with changes in luteal secretion identical to those described in early pregnancy (Illingworth et al 1990).

The model used allows description of processes occurring throughout the luteal phase, but provided the opportunity for only limited manipulation of luteal function. In future studies it may be possible to examine human luteal physiology during induced luteal regression by administration of luteolytic agents to donors prior to tissue retrieval. Alternatively the role of the luteal vasculature could be examined by administration of angiogenesis inhibitors or angiogenic growth factor antagonists prior to tissue retrieval. Ethical issues prevent the use of such compounds in human luteal donors at present, and data obtained in this study are essentially descriptive.

7.2.4 Contribution of model to work in the field

The work detailed in this thesis utilising an in vivo model of human luteal function represents a significant contribution to the field. An in vivo model of human luteal function has been developed and a large luteal tissue bank has been established, allowing description of processes involving non-steroidogenic as well as steroidogenic cells throughout the luteal lifespan.

7.3 Advances in the field during the course of these studies.

During the course of these investigations other workers have provided data complementary to our own.

7.3.1 Advances- luteal angiogenesis

Several studies have combined immunohistochemical techniques for the detection of proliferating cells and endothelial cells in order to investigate extent of angiogenesis in the corpus luteum.

Studies in the corpus luteum of the rhesus macaque (Christenson and Stouffer 1996) agree with our observation that proliferating endothelial cells are most abundant in the developing corpus luteum. Angiogenesis continues in the macaque at a lower level until a further decline at the end of the luteal phase. In parallel with our studies in the human corpus luteum hCG treatment had no effect on the level of luteal angiogenesis in the macaque. Christenson and Stouffer detected no proliferating steroidogenic cells in the rhesus monkey at any stage of luteal function, including simulated early pregnancy.

Vascular growth has been examined in the non-pregnant human corpus luteum (McClure et al 1994). In contrast to our data a fall in endothelial cell proliferation was noted during the last five days of the cycle. These authors conclude that luteal regression may be associated with declining rates of angiogenesis. The differing results may be due to the use of antibody directed against PCNA (proliferating cell nuclear antigen) to identify proliferating cells by these workers. This antibody has previously been shown to be less specific for the

identification of proliferating cells than the Ki67 used by our group (Boulton and Hodgson 1995). No other workers have examined the effects of hCG treatment on angiogenesis in the human.

7.3.2 Advances- luteal angiogenic factors

During the course of the experimental work described in this thesis VEGF expression has been examined in the corpora lutea of several species. Surprisingly, it would appear that there are marked differences in VEGF localisation and expression between species.

In ovine corpora lutea, VEGF is localised in perivascular areas (Redmer et al 1996), whereas in primates VEGF is produced in steroidogenic cells (Ravindranath et al 1992b; Gordon et al 1996; Kamat et al 1995). VEGF production in ovine corpora lutea is maximal during luteal formation, but does not decrease significantly during functional luteal regression (Redmer et al 1996). VEGF production is also maximal in the early luteal phase in monkeys (Ravindranath et al 1992b) and women (Kamat et al 1995; Gordon et al 1996) but during luteal regression a significant decrease is not noted until the onset of menses.

The importance of blood vessel formation in murine corpora lutea has been examined using AGM-470, an analogue of fumagillin with well characterised anti-angiogenic activity (Klauber et al 1997). AGM-1470 inhibits endothelial cell proliferation at concentrations which do not affect other cell types, and is potent, highly selective and non toxic. A single injection of AGM-1470 caused no decrease in serum progesterone and did not affect luteal morphology in pregnant mice. However, chronic treatment of non-pregnant rats with AGM-1470 results in a significant decrease in the size and number of corpora lutea in comparison with control animals. This implies that sustained angiogenesis is necessary for normal luteal development in the mouse.

Changing levels of angiogenic factors may affect blood vessel formation and the vascular supply of the corpus luteum, which in turn may influence luteal regression and maintenance. Further studies using specific bFGF and VEGF inhibitors are required to elucidate the role of these growth factors.

Angiogenesis in a variety of tissues is thought to be controlled by the production of angiogenic and antiangiogenic factors (Zagzag 1995; Shifren et al 1994; Wheeler et al 1995; Folkman 1995). No antiangiogenic factors have yet been detected in corpora lutea of any species but it is possible that these may also control luteal angiogenesis.

7.3.3 Advances - apoptosis

Understanding of the bcl-2 family of proto-oncogenes has expanded since the outset of this work, when bcl-2 and bax had been characterised and no other bcl-2 family members had been identified. During the course of the investigations it became apparent that bcl-2 and bax were only two members of a diverse family of structurally and functionally related proto-oncogenes. The first of these additional proto-oncogenes, bcl-x, was isolated by hybridisation to a bcl-2 probe. The bcl-x gene is alternatively spliced to produce at least two species of mRNA and protein (Boise et al 1993). Bcl-x long is highly homologous to bcl-2 and also is antiapoptotic. Bcl-x short is 63 amino acids smaller that bcl-x long and has the opposite effect by inducing apoptosis.

There are numerous other members of the bcl-2 family which may interact with one another to alter the rate of cell death. They include bak, mcl-1 bag, bad and A-1 (Farrow et al 1995; Kozopas et al 1993; Chittenden et al 1995; Lin et al 1993; Tilly 1996).

Work from other groups supports the hypothesis that bcl-2 family members may influence apoptosis in the corpus luteum. Bax has been identified in murine corpus luteum by immunoblotting but its cellular location within the gland is unknown (Krajewski et al 1994a). Bax expression is upregulated during

structural regression in bovine corpora lutea (Rueda et al 1997) but there are no descriptions of bax in the primate corpus luteum or in early pregnancy in any species. Mcl-1 has been immunolocalised in human granulosa lutein cells and bcl-x in theca lutein cells of murine corpora lutea (Krajewski et al 1994b). Expression of these proto-oncogenes at different stages of luteal function has not been described.

In order to elucidate the role that the bcl-2 family may play in regulation of luteal function we first require better understanding of interactions between the bcl-2 family members.

7.3.4 Advances- immune system

Upregulation of MCP-1 has been shown in association with macrophage influx during structural luteal regression in rats (Townson et al 1995; 1996). Recent work has demonstrated that MCP-1 production in women is inhibited by physiological levels of progesterone (Kelly et al 1997). It is possible that declining local progesterone levels during functional luteal regression allow upregulation of MCP-1 production. This may in turn trigger the influx of macrophages typically seen during structural luteal regression.

The immunoblotting and PCR studies described in this thesis have not shown significant changes in MCP-1 expression or production during functional luteal regression or maintenance. Although it is possible that the mechanism of macrophage recruitment during structural luteal regression exhibits species variation, techniques which benefit from increased sensitivity and localisation are required to further define MCP-1 expression in the human corpus luteum.

7.4 Role of the corpus luteum in the female reproductive system

The human corpus luteum is essential for human fertility.

During luteal formation and function secretion of progesterone into the bloodstream allows preparation of the endometrium for the implanting

blastocyst. In a fertile cycle, continuing progesterone secretion is essential for maintenance of the pregnancy until placental steroidogenesis later assumes this function. In the absence of a functioning corpus luteum pregnancy will not be maintained unless supported with exogenous progesterone.

In addition to this essential role in the maternal recognition of pregnancy, the corpus luteum is also an important controller of the hypothalamic pituitary ovarian axis in women. Steroid hormones secreted by the corpus luteum feed back at hypothalamic and pituitary levels to suppress FSH secretion and follicular development during the luteal phase. In a non-conception cycle falling levels of these factors at luteal regression allow resumption of FSH secretion and a new wave of folliculogenesis occurs. In order to fulfill these important roles in the regulation of human fertility the corpus luteum must develop from the dominant follicle in a short space of time, maintain a large biosynthetic capacity throughout its lifespan, be efficiently rescued in fertile cycles, and regress when conception has not occured.

7.4.1 Luteal formation

Preparation for secretion of large amounts of progesterone occurs in women prior to ovulation, with changes in the endocrinology of the dominant follicle detectable a few hours after the LH peak. The outer cells of the granulosa layer no longer convert androgen to oestrogen but instead synthesise progesterone. LH stimulates this progesterone synthesis via newly acquired LH receptors on these cells. Following ovulation a critical event in luteinisation is the invasion of the granulosa cell layer by thecal blood vessels which supply the substrates and energy necessary for progesterone biosynthesis. The development of a dense network of blood vessels to supply the steroidogenic cells of the newly formed corpus luteum is associated with strikingly high rates of angiogenesis in the gland at this time. Steroidogenic cells may control rates of luteal angiogenesis by secretion of paracrine factors such as VEGF, and rates of cell

death in the endothelial cell compartments may be low as the vasculature of the gland is established. Cytokine products of immune cells, which invade the follicle at ovulation (Brannstrom and Norman 1993) may influence rates of luteal steroidogenesis, angiogenesis or apoptosis during luteinisation.

7.4.2 Luteal regression.

Luteal regression is essential for the return of ovarian cyclicity in a non-fertile cycle, and is characterised by decreasing production of steroid and protein hormones, followed by structural regression of the gland.

In women luteal regression occurs in the absence of hCG. This contrasts with domestic species where luteal regression is triggered by secretion of PGF2α from the uterus. No such endocrine factor exists in women, and no luteolytic paracrine mediators responsible for luteolysis have been identified in human corpora lutea.

It is possible that the relatively low levels of LH which are present in the human luteal phase are insufficient to maintain the corpus luteum unless hCG stimulation is present. In the absence of sufficient gonadotrophin stimulation at the end of the luteal phase steroidogenesis may decline.

Following these initial functional regressive changes, the structure of the gland also regresses.

Functional luteal regression may also be augmented by the influx of immune cells into the gland at the end of the luteal phase. Increasing numbers of luteal immune cells may be due to secretion of chemotactic factors by steroidogenic cells during luteolysis. Immune cells may influence steroidogenesis by secretion of cytokine products or by direct cytotoxicity. In addition the decreased local blood flow noted during luteolysis (Bourne et al 1996) may result in a reduction of steroidogenic substrate availability. The studies described in this thesis detect no decrease in vascular density or luteal angiogenesis during functional

luteal regression and changes in vascular tone may be responsible for changes in luteal blood flow at this stage.

The size of the corpus luteum decreases during structural luteal regression (Zheng et al 1994). Programmed cell death has been implicated in luteolysis by studies utilising morphological and 3' end labelling techniques (Funayama et al 1996; Shikone et al 1996). Constant levels of bcl-2 and bax at this stage imply that mechanisms involved in luteolysis are dissimilar to those implicated in follicular atresia.

7.4.3 Luteal rescue

Secretion of progesterone by the human corpus luteum is a critical trigger for the maintenance of early pregnancy. Maintenance of progesterone secretion from the corpus luteum is dependant on hCG stimulation to prevent functional and stuctural luteolysis.

After stimulation by hCG luteal production of progesterone, oestrogen and inhibin increase (Illingworth et al 1990) and the size and vascular flow to the gland also increase (Alcazar et al 1996). Similar processes have been described during formation of the corpus luteum after ovulation. In contrast to the process of luteinisation the vascularity of the gland remains constant and angiogenic rates do not increase during luteal rescue. Similarly, production of angiogenic growth factors remains constant during early pregnancy. Distinct physiological processes occur during hCG stimulation in early pregnancy and during luteinisation following the LH surge.

As proliferation rates do not increase after hCG rescue of the corpus luteum it is possible that increased size of the gland may be due to hypertrophy of existing cells or to decreased rates of apoptosis. Bcl-2/bax ratios remain unchanged during luteal rescue and apoptosis may be influenced by changes in secretion of local mediators from steroidogenic or immune cells.

Immune cells are significantly less abundant in the rescued corpus luteum than during luteolysis. Lower densities of luteal macrophages in early pregnancy may remove cytokine blocks to steroid production and prevent destruction of luteal tissue.

The initial maintenance of function of the corpus luteum may be due to a direct action of hCG acting through steroidogenic cell LH receptors resulting in stimulation of progesterone biosynthesis and support of the intrauterine pregnancy. Later events in luteal maintenance may involve non-steroidogenic cells with changes in vascularity and growth factor secretion. The structure of the gland may be preserved by reduction of luteal cell death rates and by prevention of immune cell chemotaxis in the presence of hCG and progesterone.

7.5 Overview

The in vivo model of luteal function utilised in this work gives a unique insight into the possibility that the interactions between steroidogenic, immune and endothelial luteal cells may be necessary for control of luteal lifespan and hence human fertility.

REFERENCES

Abraham JA, Whang JL, Tumolo A, Mergies A, Friedman J, Gospodarowicz D and Fiddes JC (1986) Human basic fibroblast growth factor: nucleotide sequence and genomic organisation. EMBO J: 5, 2523-2528.

Akcali KC, Khan SB, and Moulton BC (1996) Effect of decidualisation on the expression of bax and bcl-2 in the rat uterine endometrium. Endocrinology: 137, 3123-3130.

Alcazar JL, Acosta MJ, Laparte L and Ruiz ML (1996) Assessment of luteal blood flow in normal and abnormal early pregnancy. J Ultrasound Med: 15, 53-56.

Alila HW and Hansel W (1984) Induction of lymphopaenia causes luteal dysfunction in cattle. Biol Reprod: 31, 671-715.

Ansari B, Coates PJ, Greenstein BD and Hall PA (1993) In situ end- labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. J Pathol: 170, 1-8.

Araki S, Shimada Y, Kaji K and Hayashi H (1990) Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. Biochem Biophy Res Commun: 168, 1194-1200.

Arici A, Oral E, Bukulmez O, Buradagunta S, Engin O and Olive DL (1996) Interleukin-8 expression and modulation in human preovulatory follicles and ovarian cells. Endocrinology: 137, 3762-3769.

Atkinson LE, Hotchkiss J, Fritz R, Surve AH, Neill JD and Knobil E (1975) Circulating levels of steroids and chorionic gonadotrophin during pregnancy in the rhesus monkey, with special attention to the rescue of the corpus luteum in early pregnancy. Biol Reprod: 12, 335-345.

Auletta FJ and Flint APF (1988) Mechanisms controlling corpus luteum function in sheep, cows, non-human primates and women especially in relation to the time of luteolysis. Endo Rev: 9, 88-105.

Auletta FJ, Caldwell BV, van Wagenen G and Morris JM (1972) The effect of postovulatory oestrogen on progesterone and prostaglandin F levels in the monkey. Contraception: 6, 411-419

Auletta FJ, Kamps DL, Pories S, Bisset J and Gibson M (1984) An intra-corpus luteum site for the luteolytic action of prostaglandin F2α in the rhesus monkey. Prostaglandins: 27, 285-298.

Auletta FJ, Paradis DK and Gibson M (1985). Luteolysis in the rhesus monkey: effect of intra-corpus luteum infusion of estrogens on luteal function. Fertil Steril: (Abs P137), p127.

Azmi TI, O'Shea JD, Bruce NW and Rodgers RJ (1984) Morphometry of the functional and regressing corpus luteum of the guinea pig. Anat Record: 210, 33-40.

Baber RJ, McSweeney MB and Gill RW (1988) Transvaginal pulsed Doppler ultrasound assessment of blood flow to the corpus luteum in IVF patients following embryo transfer. Br J Obstet Gynaecol: 95, 1226-1230.

Bagavandoss P and Wilkes JW (1991) Isolation and characterisation of microvascular endothelial cells from the developing corpus luteum. Biol Reprod: 44, 1132-1139.

Baggiolini M, Walz K and Kunkel SL (1989) Neutrophil activating peptide-1/ interleukin -8, a novel cytokine that activates neutrophils. J Clin Invest: 84 1045-1049

Baird DT (1984) The ovary. In : Austin C R and Short RV (Eds) Reproduction in Mammals, 2nd ed. Cambridge, England, Cambridge University Press.

Baird DT and Smith KB (1993) Inhibin and related peptides in the regulation of reproduction. Oxford Rev Reprod Biol: 15, 191-232.

Basilico C and Moscatelli D (1992) The FGF family of growth factors and oncogenes. Adv Cancer Res: 59, 115-165.

Basset SG, Winters SJ, Keeping HS and Zeleznik AJ (1990) Serum immunoreactive inhibin levels before and after lutectomy in the cynomolgus monkey (Maccaca fascicularis). J Clin Endocrinol Metabol: 70, 590-594.

Bassett DL (1943) The changes in vascular pattern of the ovary of the albino rat during the oestrous cycle. Am J Anat: 73, 251-291.

Bazer FW (1992) Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med: 199, 373-384.

Behrman HR and Preston SL (1989) Luteolytic actions of peroxide in rat ovarian cells. Endocrinology: 124, 2895-2900.

Behrman HR and Aten RF (1991) Evidence that hydrogen peroxide blocks hormone sensitive cholesterol transport into mitochondria of rat luteal cells. Endocrinology: 128, 2958-66.

Behrman HR, Yoshinaga K and Greep R (1971). Extraluteal effects of prostaglandins. Ann New York Acad Sci: 180, 426-435.

Behrman HR, Endo T, Aten RF and Musicki B (1993) Corpus luteum function and regression. Reprod Med Rev: 2, 153-180.

Beling CG, Stewart LM and Markham SM (1970) Functional activity of the corpus luteum following hysterectomy. J Clin End Metabol: 30, 30-39.

Bennegard B, Hahlin M, Wennberg E, Noren H (1991) Local luteolytic effect of prostaglandin F2α in the human corpus luteum. Fertil Steril: 56, 1070-1076.

Best CL, Pudney J, Welch WR, Burger N and Hill JA (1996) Localisation and characterisation of white blood cell populations within the human ovary throughout the menstrual cycle and menopause. Hum Reprod: 11, 790-797.

Bhattacharya AN, Dierschke DJ, Yamaji T and Knobil E (1972) The pharmocologic blockade of the circhoral mode of LH secretion in the ovariectomized monkey. Endocrinology: 90, 778-786.

Bikfalvi, A, Klein S, Pintucci G and Rifkin DB (1997) Biological roles of fibrobast growth factor-2. Endo Rev: 18, 26-46.

Bissonette RP, Echeverri F, Mahboubi A and Green DR (1992) Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature: 359, 552-554.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindstern T, Turka LA, Mas X, Nunez G and Thomson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell: 74, 597-608.

Bortner CD, Oldenburg NBE and Cidlowski JA (1995) The role of DNA fragmentation in apoptosis. Trends Cell Biol: 5, 21-26.

Boulton RA and Hodgson HJF (1995) Assessing cell proliferation -a methodological review. Clin Science: 88, 119-130.

Bourne TH, Hastrom H-G, Hahlin M, Josefsson B, Granberg S, Hellberg P, Hamberger L and Collins WP (1996) Ultrasound studies of vascular and morphologic changes in the human corpus luteum during the menstrual cycle. Fertil Steril: 65, 753-758.

Bowen ID (1993) Apoptosis or programmed cell death? Cell Biol Int: 17, 365-380.

Braden TD, Gamboni F and Niswender GD (1988) Effects of prostaglandin F2α induced luteolysis on the populations of cells in the ovine corpus luteum. Biol Reprod: 39, 245-253.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Analytical Biochemistry: 72, 248-254.

Brannstrom M and Norman RJ (1993) Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. Hum Reprod: 8, 1762-1775.

Brannstrom M, Mayrhofer G and Robertson S (1993) Localisation of leucocyte subsets in the rat ovary during the periovulatory period. Biol Reprod: 48, 277-286.

Brannstrom M, Pascoe V, Norman, RJ and McClure N (1994a) Localisation of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. Fertil Steril: 61, 488-495.

Brannstrom M, Giesecke L, van den Heuvel CJ, Moore IC and Robertson SA (1994b) Leukocyte subpopulations in the rat corpus luteum during pregnancy and pseudopregnancy. Biol Reprod: 50, 1161-1167

Brown LF, Berse BE, Tognazzi K, Manseau EJ, Van der Water L, Senger DR and Dvorak HF (1992) Vascular permeability factor mRNA and protein expression in human kidney. Kidney Int: 42, 1457-1461.

Bruce NW and Moor RM (1976) Capillary blood flow to ovarian follicles, stroma and corpora lutea of anaesthetised sheep. J Reprod Fertil: 46, 299-304.

Bruce NW, Meyer GT and Dharmarajan AM (1984) Rate of blood flow and growth of corpora lutea of pregnancy and of previous cycles throughout pregnancy in the rat. J Reprod Fertil: 71, 445-452.

Bugler B, Amalric F and Prats H (1991) Alternative initiation of translation determines cytoplasmic or nuclear localisation of basic fibroblastic growth factor. Mol Cell Biol: 11, 573-577.

Butcher RL, Collins WE and Fugo NW (1974) Plasma concentrations of LH, FSH, prolactin, progesterone and oestradiol throughout the 4-day estrous cycle of the rat. Endocrinology: 96, 1704-1708.

Caldwell BV, Rochell VE, Pang CY, Anderson GG and Behrman HR (1980) Comparative study of high dose chorionic gonadotrophin on the human and rat corpus luteum and the effects of gonadotrophin releasing hormone on human luteal function. Am J Obstet Gynaecol: 136, 458-464.

Campbell BK, Gordon BM, Tsonis CG and Scaramuzzi RJ (1992) The effect of immunisation of ewes against inhibin on plasma FSH, ovarian oestradiol secretion and folllicular development. An Reprod Sci: 28, 936-938.

Carmeliet P, Ferreira V, Breier G, Pollyfeyt S, Kiekens L, Gerlenstein M, Fahrig M, Vanderhoeck A, Harpal K, Eberhardt C, Declerq C, Pualing J, Moons L, Collen D, Risua W and Nagy A (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature: 380, 435-439.

Carson SA (1996) Recurrent fetal wastage: non genetic causes: In Queenan, JT and Hobbins JC (Eds). Protocols for high risk pregancies, 3rd ed. Cambridge, MA, USA, Blackwell, 469-477.

Challis JRG, Calder AA, Dilley A, Forster CS, Hillier K, Hunter DJ MacKenzie TZ and Thorburn GD (1976) Production of prostaglandins E and F2α by corpora lutea, corpora albicantes and stroma of the human ovary. J Endocrinol: 68, 401-408.

Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI and Guild BC (1995) Induction of apoptosis by the Bcl-2 homologue Bak. Nature: 374, 733-736.

Chomczynski P and Saatchi N (1987) Single step method of RNA isolation by acid guanidium thiocynate-phenol-chloroform extraction. Anal Biochem: 162, 156-159.

Christenson LK and Stouffer RL (1996) Proliferation of microvascular endothelial cells in the primate corpus luteum during the menstrual cycle and in simulated early pregnancy. J Clin Endocrinol Metabol: 137, 367-374.

Chun S-Y, Billig H, Tilly JI, Furata I, Tsafriri A and Hsueh AJW (1994) Gonadotrophin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor. Endocrinology: 135, 1845-1853.

Clement PB (1987) Histology of the ovary. Am J Surg Pathol: 11, 277-303.

Conley AJ, Kaminski MA, Dubowski SA, Jablonka-Shariff A, Redmer DA and Reynolds LP (1995) Immunohistochemical localisation of 3β-hydroxysteroid dehydrogenase and P450 17α-hydroxylase during follicular and luteal development in pigs, sheep and cows. Biol Reprod: 52, 1081-1094.

Connolly DT, Heuvelman DM, Nelson R, Olander JU, Eppley BL, Delfino JJ, Seigel NR, Leimgruber RM and Feder J (1989a) Tumour vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest: 84, 1470-1478.

Connolly DT, Olander JV, Heuvelman D, Nelson R, Mansell K, Siegel N, Haymore BL, Leimgruber RM and Feder J (1989b) Human vascular permeability factor. Isolation from U937 cells. J Biol Chem: 254, 20017-20024.

Cooper J, Sharkey A, McLaren J, Charnock-Jones D and Smith S (1995) Localisation of vascular endothelial growth factor and its receptor flt in human placenta and decidua by immunohistochemistry. J Reprod Fertil; 105, 205-213.

Corner GW (1956) The histological dating of the human corpus luteum of menstruation. Am J Anat: 98, 377-401.

Corner GW and Allen WH (1929) Physiology of corpus luteum: production of special uterine reaction (progestational proliferation) by extracts of corpus luteum. Am J Physiol: 88, 326-333.

Coucouvanis EC, Sherwood SW, Carswell-Crumpton C, Spack EG and Jones PP (1993) Evidence that the mechanism of prenatal germ cell loss in the rat is apoptosis. Exp Cell Res: 209, 238-247.

Cross SS, Start RD, Smith JHF(1990) Does delay in fixation affect the number of mitotic figures in procressed tissues? J Clin Pathol: 43, 597-599.

Cullinan-Bove K and Koos RD (1993) Vascular endothelial growth factor/ vascular permeability factor in the rat uterus: rapid stimulation by oestrogen correlates with oestrogen -induced increases in uterine capillary permeability and growth. Endocrinology: 133, 829-837.

D'Amore PA and Thomson RW (1987) Mechanisms of angiogenesis. Ann Rev Physiol :49, 453-464.

de Graaf R (1672) De mulerierum organis generationi inservientibus tractatus novus. Jocelyn HD, Setchell BP (trans.) J Reprod Fertil (Suppl) 1972.

de Vries C, Escobedo JA, Ueno H, Houck KA, Ferrara N and Williams LT (1992) The tms-like tyrosine kinase; a receptor for vascular endothelial growth factor. Science: 255, 989-991.

Deichert U, Albrand-Thielman C and van der Sandt M (1996) Doppler-sonographic pelvic blood flow measurements and their prognostic value in terms of luteal phase and implantation. Hum Reprod: 11, 1591-1593.

Deikman MA, O'Callaghan P, Nett T and Niswender GD (1978) Effect of prostaglandin F2α on the numbers of LH receptors in ovine corpora lutea. Biol Reprod: 19, 1010-1013.

Denekamp J (1984) Vasculature as a target for tumour therapy. In: Hammerson F and Hudlicka O (Eds) Progress in applied microcirculation, Vol 4, Basel, Switzerland, Karger, 28-33.

Dennefors BL, Sjorgen A and Hamberger L (1982) Progesterone and adenosine 3' 5'-monophosphate formation by human corpora lutea of different ages: influence of human chorionic gonadotrophin and prostaglandins. J Clin Endocrinol Metabol: 55, 102-107.

Dharmarajan AM, Bruce NW and Meyer GT (1985) Quantitative ultrastructural characteristics relating to transport between luteal cell cytoplasm and blood in the corpus luteum of the pregnant rat. Am J Anat: 172, 87-99.

Djahanbakhch O, McNeilly AS, Hobson BM and Templeton AA (1981) A rapid luteinising hormone radioimmunoassay for the prediction of ovulation. Br J Obstet Gynaecol: 88, 1016-1020.

Doraiswamy V, Grazul-Bilska AT, Ricke WA, Redmer DA and Reynolds LP (1995a) Immunoneutralisation of angiogenic activity from ovine corpora lutea (CL) with antibodies against fibroblast growth factor (FGF)-2 and vascular endothelial growth factor (VEGF). Biol Reprod: 52, Supplement 1, 112.

Doraiswamy V, Grazulbilska AT, Redmer DA, Killilea SD and Reynolds LP (1995b). Expression of fibroblast growth factor receptors (FGFR) in ovine corpora lutea. Proceedings of the 35th Annual Meeting: Am Soc Cell Biol, Washington DC.

Doraiswamy V, Moor RM, Dai Y, Reynolds LP and Redmer DA (1996) Immunolocalisation of vascular endothelial growth factor (VEGF) in ovine corpora lutea (CL) throughout the oestrous cycle. Biol Reprod:54, Supplement 1, 34.

Duke RC and Cohen JJ (1990) Il-2 addiction: withdrawal of growth factor activates a suicide programme in dependent T-cells. Lymphokine Res: 5, 289-299.

Duncan WC, Illingworth PJ and Fraser HM (1996a) Expression of tissue inhibitor of metalloproteinases 1 (TIMP-1) in the primate ovary during induced luteal regression. J Endocrinol: 151, 203-213.

Duncan WC, Mc Neilly AS and Illingworth PJ (1996b) Expression of tissue inhibitor of metalloproteinases-1 in the human corpus luteum after luteal rescue. J Endocrinol: 148, 59-67.

Duncan WC, McNeilly AS and Illingworth PJ (1996c). Differential localisation of messenger RNAs of metalloproteinases and their inhibitors in the human corpus luteum. Biol Reprod: 54, 357-361.

Duncan WC, McNeilly AS and Fraser HM (1996d) Luteinising hormone receptor in the human corpus luteum- lack of downregulation during maternal recognition of pregnancy. Human Reproduction 11, 2291-2297.

Duncan WC, Rodger FE and Illingworth PJ (1998) The human corpus luteum: reduction in macrophages during maternal recognition of pregnancy. Hum Reprod; in press.

Duvall E, Wyllie AH and Morris RG (1985) Macrophage recognition of cells undergoing programmed cell death. Immunology: 56, 351-358.

Dvorak HF (1986) Tumours: wounds that do not heal. Similarity between tumour stroma generation and wound healing. New Engl J Med: 315, 1650-1658.

Dvorak HF, Harvey VS, Estrella P, Brown LF, McDonagh J and Dvorak AM (1987) Fibrin containing gels induce angiogenesis: implications for tumour stroma generation and wound healing. Lab Invest: 57, 673-686.

Ellinwood WE and Resko JA (1983) Effect of inhibition of oestrogen synthesis during the luteal phase on function of the corpus luteum in rhesus monkeys. Biol Reprod: 28, 636-644.

Ellinwood WE, Nett TM, Niswender GD and Jones RE (1978) Ovarian vasculature: structure and function. In: Jones RE (Ed) The vertebrate ovary, New York, USA; Plenum.

Emi N, Kanzak H, Yoshida M , Takakura K, Okamoto N, Imai K and Mori T (1991) Lymphocytes stimulate progesterone production by cultured human granulosa lutein cells. Am J Obstet Gynaecol: 165, 1469-1474.

Endo T, Aten RF, Leykin L and Behrman HR (1993) Hydrogen peroxide evokes antisteroidogenic and antigonadotrophic actions in human granulosa lutein cells. J Clin Endocrinol Metabol: 76, 337-342.

Erickson AE, Cousens LS, Weaver LH and Mattews BW (1991) Three dimensional structure of human basic fibroblast growth factor. Proc Nat Acad Sci USA: 88, 3341-3445.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brocks M, Waters CM, Penn LB and Hancock DC (1992) Induction of apoptosis in fibroblasts by c-myc protein. Cell :69, 119-128.

Eyster KM, Ottobre JS and Stouffer RL (1985) Adenylate cyclase in the corpus luteum of the rhesus monkey (III) Changes in basal and gonadotrophin-sensitive activities during the luteal phase of the menstrual cycle. Endocrinology:117, 1571-1577.

Fairchild DL, Benyo D and Pate JL (1992) Tumour necrosis factor alpha alters bovine luteal cell synthetic capacity and viability. Endocrinology: 130, 854-860.

Fandini A, Harrington EA and Evan GI (1992) Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature: 359. 554-556.

Farrow SN, White JHM, Martinou I, Raven T, Punn KT, Grisham CJ, Martinou JC and Brown R (1995) Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. Nature: 374, 731-733.

Ferrara N and Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun: 161, 851-858.

Ferrara N and Davis-Smith T (1997) The biology of vascular endothelial growth factor. Endo Rev: 18, 4-25.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ and Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of VEGF. Nature: 380, 439-442.

Fincher KB, Bazer FW, Hansen PJ, Thatcher WW and Roberts RM (1986) Proteins secreted by the sheep conceptus suppress induction of uterine prostaglandin F2α release by oestradiol and oxytocin. J Reprod Fertil: 76, 425-433.

Flemming W (1885) Uber die Bildung von Richtungsfiguren in Saugethiereiern beim Untergang Graaf'sher Follikel. Arch Anat Entw Gesch 1885, 221-224.

Florkiewicz RZ and Somer A (1989) Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non- AUG codons. Proc Nat Acad Sci USA: 86, 3978-3981.

Florkiewicz RZ, Baird A and Gonzalez AM (1991) Multiple forms of bFGF: differential nuclear and cell surface localisation. Growth Factors: 4, 265-275.

Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Med: 1, 27-31.

Folkman J and Cotran R (1976) Relation of vascular proliferation to tumour growth. Int Rev Exp Pathol: 16, 207-248.

Folkman J and Klagsburn M (1987) Angiogenic factors. Science: 233, 442-447.

Fong G-H, Rossant J, Gertenstein M and Breitman M (1995) Role of flt-1 receptor tyrosine kinase in regulation of assembly of vascular endothelium. Nature: 376, 62-66.

Frankel L (1903) Die Function des Corpus Luteum. Arch. Gynaekol: 68, 438-447.

Fraser HM and Lunn SF (1993) Does inhibin have an endocrine function during the menstrual cycle? Trends Endocrinol Metabol: 4, 187-194.

Fraser HM and Tsonis CG (1994) Manipulation of inhibin during the luteal-follicular phase transition of the primate menstrual cycle fails to affect FSH secretion. J Endocrinol: 142, 181-186.

Fraser HM, Baird DT, McRae GI, Nestor JJ and Vickery BH (1985) Suppression of luteal progesterone secretion in the stumptailed macaque by an antagonist analogue of luteinizing-hormone releasing hormone. J Endocrinol: 104, R1-R4.

Fraser HM, Robertson DM and de Kretser DM (1989) Immunoreactive inhibin concentrations in serum throughout the menstrual cycle of the macaque: suppression of inhibin during the luteal phase after treatment with an LHRH agonist. J Endocrinol:121, R9-R12.

Fraser HM, Lunn SF, Cowen GM and Illingworth PJ (1995) Induced luteal regression in the primate: evidence for apoptosis and changes in c-myc protein. J Endocrinol: 147, 131-137.

Fritz MA and Speroff L (1982) The endocrinology of the menstrual cycle: the interaction of folliculogenesis and neuroendocrine mechanisms. Fertil Steril: 38, 509-529

Fukuoka M, Yasuda K, Taii S, Takakura K and Mori T (1989) Interleukin-1 stimulates growth and inhibits progesterone secretion in the culture of porcine granulosa cells. Endocrinology: 124, 844-890.

Fukuoka M, Yasuda K, Emi N, Fujiwara H, Iwai M, Takawa K, Kanzaki H and Mori T (1992) Cytokine modulation of progesterone and estradiol secretion in cultures of luteinised human granulosa cells. J Clin Endocrinol Metabol: 75, 254-258.

Funayama Y, Sasano H, Suzuki T, Tamura M, Fukaya T and Yajima A (1996) Cell turnover in normal cycling human ovary. J Clin Endocrinol Metabol: 81, 828-834.

Garrido C, Saule S and Gospodarowicz D (1993) Transcriptional regulation of vascular endothelial growth factor expression in ovarian bovine granulosa cells. Growth Factors: 8, 109-117.

Gavrieli Y, Sherman Y and Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol: 119, 493-501.

Gerdes J, Schlueter C, Wohlenberg C and Gerlach C (1991) Immunobiochemical and molecular biological characterisation of the cell proliferation- associated nuclear antigen that is defined by monoclonal antibody Ki67. Am J Pathol: 138, 867-873.

Gerdes U, Gafvels M, Bergh A and Cajander S (1992) Localised increases in ovarian vascular permeability and leukocyte accumulation after induced ovulation in rabbits. J Reprod Fertil: 95, 539-550.

Ghinea N, Hai MTV, Groyer-Picard MT and Milgrom E (1994) How protein hormones reach their target cells. Receptor mediated transcytosis of hCG through endothelial cells. J Cell Biol: 125, 87-97.

Gillim SW, Christensen KA and McLennan CE (1969) Fine structure of the human menstrual corpus luteum at its stage of maximum secretory activity. Am J Anat : 126, 409-428.

Glock JL, Blackman JA, Badger GJ and Brumsted JR (1995) Prognostic significance of morphologic changes of the corpus luteum by transvaginal ultrasound in early pregnancy. Obstet Gynaecol: 85, 37-41.

Gompel A, Sabourin JC, Martin A, Yaneva H, Audouin J, Decroix Y and Poitout P (1994) Bcl-2 expression in normal endometrium during the menstrual cycle. Am J Pathol: 144, 1195-1202.

Gordon JD, Mesiano S, Zaloudek CJ and Jaffe RB (1996) Vascular endothelial growth factor localisation in the human ovary and fallopian tubes: Possible role in reproductive function and ovarian cyst formation. J Clin Endocrinol Metabol: 81, 353-359.

Gore BZ, Caldwell BV and Speroff L (1973) Oestrogen induced human luteolysis. J Clin Endocrinol Metabol: 36, 613-620.

Gospodarowicz D and Thakral KK (1978) Production of a corpus luteum angiogenic factor responsible for proliferation of capillaries and neovascularisation of the corpus luteum. Proc Nat Acad Sci USA: 75, 847-851.

Gospodarowicz D, Cheng J, Lui GM, Baird A, Esch F and Bahlen P (1985) Corpus luteum angiogenic factor is related to fibrobast growth factor. Endocrinology: 6, 2383-2391.

Gospodarowicz D, Ferrara N, Schweigerer L and Nuefeld G (1987) Structural charaterisation and biological functions of fibroblast growth factor. Endo Rev: 8, 95-114.

Gospodarowicz D, Abraham JA and Schilling J (1989) Isolation and characterisation of a vascular endothelial cell mitogen produced by pituitary derived folliculostellate cells. Proc Nat Acad Sci USA: 86, 7311-7315.

Graem N and Helweg-Larsen K (1979) Mitotic activity and delay in fixation of tumour tissue. Acta Pathol Microbiol Scand: 87, 375-378.

Graper L (1914) Eine neue Anschauung uber physiologische Zellausschaltung. Arch Zellforsch: 12, 373-394.

Grazul-Bilska AT, Redmer DA and Reynolds LP (1991) Secretion of angiogenic activity and progesterone by ovine luteal cell types in vitro. J Anim Sci: 69, 2099-2107.

Grazul-Bilska AT, Redmer DA, Killilea SD, Kraft KC and Reynolds LP (1992) Production of mitogenic factor(s) by ovine corpora lutea throughout the estrous cycle. Endocrinology: 130, 3625-3632.

Grazul-Bilska AT, Redmer DA, Killilea SD, Zheng J, and Reynolds LP (1993) Initial characterisation of endothelial mitogens produced by bovine corpora lutea from the estrous cycle. Biochem Cell Biol: 71, 270-277.

Grazul-Bilska AT, Redmer DA, Killilea SD and Reynolds LP (1995) Initial characterisation of mitogenic activity of ovine corpora lutea from early pregnancy. Growth Factors: 12, 131-144.

Gu Y, Jow GM, Moulton BC, Lee C, Sensibar JA Parke-Sarge OK, Chen JJ and Gibori G (1994) Apoptosis in decidual tissue regression and reorganisation. Endocrinology: 135, 1272-1279.

Hall PF (1985) Trophic stimulation of steroidogenesis: in search of the elusive trigger. Rec Prog Hormone Res: 41, 1-39.

Halme J, Hammond MG, Syrop CH and Talbert LM (1985) Peritoneal macrophages modulate human granulosa-luteal cell progesterone production. J Clin Endocrinol Metabol: 61, 912-916.

Harrington EA, Bennet MR, Fandini A and Evan GI (1994) C-myc induced apoptosis in fibroblasts is inhibited by cytokines. Eur Molec Biol J: 13, 3286-3295.

Hearn JP and Webley GE (1987) Regulation of the corpus luteum of early pregnancy in the marmoset monkey- local interactions of luteotrophic and luteolytic hormones in vivo and their effects on the secretion of progesterone. J Endocrinol: 114, 231-239.

Hearn JP, Webley GE and Gidley-Baird AA (1991) Chorionic gonadotrophin and embryo-maternal recognition during the peri-implantation period in primates. J Reprod Fertil: 92, 497-509.

Heder G, Jakob W, Halle W, Mauersberger B, Rambach G Jentzsch KD and Oehme P (1979) Influence of porcine corpus luteum extract on DNA synthesis and proliferation of cultivated fibroblasts and endothelial cells. Exp Pathol: 17, 493-497.

Hengartner MO and Horvitz HR (1994) Activation of C. elegans cell death protein ced-9 by an amino-acid substitution in a domain conserved in bcl-2. Nature: 369, 318-320.

Hild-Petito SA, Shiigi SM and Stouffer RL (1989) Isolation and characterisation of cell populations from the monkey corpus luteum of the menstrual cycle. Biol Reprod: 40, 1075-1085.

Hockenbery D, Nunez G, Milliman C, Schreiber RD and Korsmeyer SJ (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature: 348, 334-336.

Hockenbery DM, Zutter M, Hickey W, Nahm M and Korsmeyer SJ (1991) Bcl-2 protein is topographically restricted in tissues characterised by apoptotic cell death. Proc Nat Acad Sci USA: 88, 6961-6965.

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL and Korsmeyer SJ (1993) Bcl-2 functions in an anti-oxidant pathway to prevent apoptosis. Cell: 75, 241-251.

Hoff QD, Quigley ME and Yen SSC (1983) Hormonal dynamics at mid-cycle- a re-evaluation. J Clin Endocrinol Metabol: 57, 792-796.

Holmgren L, O'Reilly MS and Folkman J (1995) Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nature Med: 1, 149-153.

Horton EW and Poyser NL (1976) Uterine luteolytic hormone: A physiological role for prostaglandin F2α. Physiol Rev: 56, 595-561.

Hosang K, Knoke I, Klaudiny J, Wempe F, Wuttke W and Scheit KH (1994) Porcine luteal cells express monocyte chemoattractant protein-1 (MCP-1)-analysis by polymerase chain reaction and cDNA cloning. Biochem Biophys Res Comm: 199, 962-968.

Houmard BS and Ottobre JS (1989) Progesterone and prostaglandin production by primate luteal cells collected at various stages of the luteal phase- modulation by calcium ionophore. Biol Reprod: 41, 401-408.

Hutchison JS and Zeleznik AS (1984) The rhesus monkey corpus luteum is dependant on pituitary gonadotrophin secretion throughout the luteal phase of the menstrual cycle. Endocrinology: 115, 1780-1786.

Illingworth PJ, Reddi K, Smith K and Baird DT (1990) Pharmacologic "rescue" of the corpus luteum results in an increased inhibin production. Clin Endocrinol: 33, 323-332.

Illingworth PJ, Reddi K, Smith KB and Baird DT (1991). The source of inhibin secretion during the human menstrual cycle. J Clin Endocrinol Metabol: 73, 667-673.

Illingworth, PJ, Fraser HM, Young FM, Lunn SF and Cowen GM (1994) Localisation of c-myc in the human corpus luteum. The Endocrine Society 76th Annual Meeting: Abstract 969.

Illingworth PJ, Groome NP, Duncan WC, Grant V, Tovanabutra S, Baird DT and McNeilly AS (1996) Measurement of circulating inhibin forms during the establishment of pregnancy. J Clin Endocrinol Metabol:81, 1471-1475.

Ito WD, Arras M, Winkler B, Scholz D, Schaper J and Schaper W (1997) Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. Circ Res: 80, 829-837.

Jablonka-Shariff A, Grazul-Bilska AT, Redmer D, and Reynolds LP (1993) Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle. Endocrinology: 133, 1871-1879.

Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC and Raff MC (1993) Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature: 361, 365-367.

Jacobson MD, Burne JF and Raff MC (1994) Programmed cell death and bcl-2 protection in the absence of a nucleus. EMBO J: 1899-1910.

Jakeman LB, Winer J, Bennet GL, Altar CA and Ferrara N (1992) Binding sites for vascular endothelial growth factor are localised on endothelial cells in adult rat tissues. J Clin Invest: 89, 244-253.

Jakeman LB, Armanini M, Phillips HS and Ferrara N (1993) Developmental expression of binding sites and mRNA for vascular endothelial growth factor

suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology: 133, 848-849.

Jakob W, Jentzsch KD, Mauersberger B and Oehme P (1977) Demonstration of angiogenesis activity in the corpus luteum of cattle. Exp Pathol: 13, 231-236.

Jeffcoate N (1987) Tumours of the ovary. In: VR Tindall (Ed) Jeffcoate's Principles of Gynaecology, 5th Ed, London, UK, Butterworth Press, 450-482.

Ji T, Slaughter RG, Ellis JA, Ji TH and Murdoch WJ (1991) Analyses of ovine corpora lutea for tumour necrosis factor mRNA and bioactivity during prostaglandin induced luteolysis. Mol Cell Endocrinol: 81, 77-80.

Juengel JL, Garverick HA, Johnson AL, Youngquist RS and Smith MF (1993) Apoptosis during luteal regression in cattle. Endocrinology: 132, 249-254.

Kamat BR, Brown LM, Manseau EJ,Senger DR and Dvorak HF (1995) Expression of vascular permeability factor/ vascular endothelial growth factor by human granulosa and theca lutein cells. Am J Pathol: 14, 157-165.

Kamel OW, Franklin WA, Ringus JC and Meyer JS (1989) Thymidine labelling index and Ki67 growth fraction in lesions of the breast. Am J Pathol: 134, 107-113.

Kane DJ, Sarafian TA, Anton R, Hahn H, Grolla EB, Valentine Ts, Ord T and Bredesen DE (1993) Bcl-2 inhibition of neural death-decreased generation of reactive oxygen species. Science: 262, 1274-1277.

Karim SSM and Hillier K (1979) Prostaglandins in the control of animal and human reproduction. Br Med Bull :35, 173-180.

Karsch FJ and Sutton GP 1976. An intra ovarian site for the luteolytic action of estrogen in the rhesus monkey Endocrinology: 98, 553-561.

Kelly RW, Carr GG and Riley SC (1997) The inhibition of synthesis of a beta chemokine, monocyte chemotactic protein-1 (MCP-1) by progesterone. Biochem Biophys Res Comm: 239, 557-561.

Kerr JFR, Wyllie AH and Currie AR (1972) Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer: 26, 239-257.

Khan-Dawood FS, Huang J-C and Dawood MY (1989) Effect of human chorionic gonadotrophin and prostaglandin F2α on progesterone production by human luteal cells. J Steroid Biochem: 33, 941-947.

Klauber N, Rohan R, Flynn E and D'Amato RJ (1997) Critical components of the female reproductive pathway are suppressed by the angiogenesis inhibitor AGM-1470. Nature Med: 3, 443-446.

Klein, NA, Illingworth PJ, Groome NP, McNeilly AS, Battaglia DE and Soules MR (1996) Decreased inhibin B secretion is associated with the monotropic FSH rise in older, ovulatory women: A study of serum and follicular fluid levels of dimeric inhibin A and B in spontaneous menstrual cycles. J Clin Endocrinol Metab: 81, 2742-2745.

Knobil E (1974) On the control of gonadotrophin secretion in the rhesus monkey. Rec Prog Hormone Res: 30, 1-46

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner Um, Elner SG and Streiter RM (1992) Interleukin-8 as a macrophage derived mediator of angiogenesis. Science: 258, 1798-1801.

Koh EAT, Illingworth PJ, Duncan WC and Critchley HOD (1995) Immunolocalisation of bcl-2 protein in human endometrium in the menstrual cycle and simulated early pregnancy. Hum Reprod: 10, 1557-1563.

Koos RD (1989) Potential relevance of angiogenic factors to ovarian physiology. Sem Reprod Endocrinol: 7, 29-40

Koos RD and LeMaire WJ (1983) Evidence for an angiogenic factor from rat follicles. In: Greenwald GS and Terranova PF (Eds) Factors regulating ovarian function. New York, Raven Press, 191-195.

Korda AR, Shutt DA, Smith ID, Shearman RP and Lyneham RC (1975) Assessment of possible luteolytic effect of intraovarian injection of prostaglandin F2α in the human. Prostaglandins: 9, 443-449.

Kozopas KM, Yang T, Buchan HI , Zhou P and Craig RW (1993) Mcl-1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to bcl-2. Proc Nat Acad Sci USA: 90, 35216-3520.

Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG and Reed JC (1994a) Immunohistochemical determination of in vivo distribution of bax, a dominant inhibitor of bcl-2. Am J Pathol: 145, 1323-1335.

Krajewski S, Krajewska M, Shabaik A, Wang HG, Irie S, Fong L and Reed JC (1994b) Immunohistochemical analysis of in vivo patterns of bcl-x expression. Cancer Res: 54, 5501-5507.

Krajewski S, Bodrug S, Krajewska M, Shabaik A, Gascoyne R, Berean K and Reed JC (1995) Immunohistochemical analysis of mcl-1 protein in human tissues. Am J Pathol: 46, 1309-1319.

Kuwubara K, Ogawa S, Matsumoto M , Koga S, Clauss M, Pinsky DT, Lyn P, Leavy J, Witte L and Joseph-Silverstein J (1995) Hypoxia mediated induction of acidic/basic fibroblast growth factor and platelet derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. Proc Nat Acad Sci USA: 92, 4606-4610.

Kyprianou N, English HF and Isaacs JT (1990) Programmed cell death during regression of PC82 human prostate cancer following androgen ablation. Cancer Res: 50, 3748-3753.

Le Maire WJ, Rice BF and Savard K (1968) Steroid formation in the human ovary. V. Synthesis in vitro in corpora lutea during the reproductive cycle. J Clin Endocrinol Metabol: 28, 1249-56.

Lea RG, Al-Sharek H, Tulppala M and Critchley HOD (1997) The immunolocalisation of bcl-2 at the maternal-fetal interface in healthy and failing pregnancies. Hum Reprod: 12, 153-158.

Lee CY, Coulam CG, Jiang NS and Ryan RJ (1977) Receptors for luteinising hormone in human corpora luteal tissue. J Clin Endocrinol Metabol: 36, 148-152.

Lei ZM, Chegini N and Rao CV (1991) Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol Reprod: 44, 1148-1156.

Leung DW, Cachianes G, Kuang W-J, Goeddel DV and Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science: 246, 1306-1309.

Levy AP, Levy NS, Wegner S and Goldberg MA (1995) Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J Biol Chem: 270, 13333-13340.

Li TC, Rogers AW, Dockery P, Lenton EA and Cooke ID (1988) A new method of histologic dating of human endometrium in the luteal phase. Fertil Steril: 50, 52-60.

Lin EY, Orlofski A, Bergar M and Prystowsky MB (1993) Characterisation of A1, a novel hematopoietic- specific early response gene with sequence similarlty to bcl-2. J Immunol: 151, 1979-1988.

Lowe SW, Ruley HE, Jacks T and Housman DE (1993a) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell: 74, 957-967.

Lowe SW, Schmitt EM, Smith EW, Osborne BA and Jacks T (1993b) p53 is required for radiation induced apoptosis in mice thymocytes. Nature: 362, 847-849.

Luciano AM, Pappalardo A, Ray C and Peluso JJ (1994) Epidermal growth factor inhibits large granulosa cell apoptosis by stimulating progesterone synthesis and regulating the distribution of intracellular free calcium. Biol Reprod: 51, 646-654.

Lynch MP, Nawas S and Gerschenson LE (1986) Evidence for soluble factors regulating cell death and cell proliferation in primary cultures of rat endometrium. Proc Nat Acad Sci USA: 83, 4784-4788.

Maas S, Jarry H, Teichmann A, Rath W, Kuhn W and Wuttke W (1992) Paracrine actions of oxytocin, prostaglandin F2α and estradiol within the human corpus luteum. J Clin Endocrinol Metabol: 74, 306-312.

Mais V, Kazer RR, Cetel NS, Rivier J, Vale W and Yen SSC (1986) The dependency of folliculogenesis and corpus luteum function on pulsatile gonadotropin secretion in cycling women using a gonadotrophin-releasing hormone antagonist as a probe. J Clin Endocrinol Metabol: 62, 1250-1255.

Manjo G and Joris I (1995) Apoptosis, oncosis, and necrosis. Am J Pathol: 146, 3 -15.

Margolin Y, Aten RF and Behrman HR (1990) Antigonadotrophic and antisteroiodgenic actions of peroxide in rat granulosa cells. Endocrinology: 127, 245-250.

McClure N, McPherson AM, Healy DL, Wreford N and Rogers PAW (1994) An immunohistochemical study of the vasculature of the human Graafian follicle. Hum Reprod: 9, 1401-1405.

McComb RD, Jones TR, Pizzo SV and Bigner DD (1982) Specificity and sensitivity of immunohistochemical detection of factor-VIII von Willebrand factor antigen in formalin-fixed paraffin embedded tissue. J Histochem Cytochem: 30, 371-377.

McCracken JA, Schramm W, Barcikowski B and Wilson L (1981) The identification of prostaglandin F2α as a uterine luteolytic hormone in the sheep and the endocrine control of its synthesis. Acta Vet Scad: 77 (Suppl), 71-88.

McFarland KC, Sprengel R, Phillips HS, Kohler M, Rosemblit N, Nicoloics K, Segaloff DC and Seeberg PH (1989) Lutropin-chorionic gonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science: 245, 525-528.

McKay DG, Pinkerton JHM, Hertig AT and Danziger S (1961) The adult human ovary: a histochemical study. Obstet Gynaecol: 18, 13-39.

McLaren J, Prentice A, Charnock-Jones DS, Sharkey M and Smith SK (1997) Immunolocalisation of the apoptosis regulating proteins Bcl-2 and Bax in human endometrium and isolated peritoneal fluid macrophages in endometriosis. Hum Reprod: 12, 146-152.

McNeilly AS, Kerin J, Swanston IA, Bramley TA and Baird DT (1980) Changes in the binding of human chorionic gonadotrophin/luteinising hormone, follicle stimulating hormone and prolactin to human corpora lutea during the menstrual cycle and pregnancy. J Endocrinol: 87, 315-325.

Meyer GT and Bruce NW (1980) Quantitative cell changes and vascularisation in the early corpus luteum of the pregnant rat. Anat Rec: 197, 369-374.

Meyer GT and McGeachie JK (1988) Angiogenesis in the developing corpus luteum of pregnant rats: a stereologic and autoradiographic study. Anat Rec: 222, 18-25.

Meyer JS (1982) Cell kinetic measurements from human tumours. Hum Pathol: 12, 874-7.

Michael AE, Abayasekara DRE and Webley GE (1994) Cellular mechanisms of luteolysis. Mol Cell Endocrinol: 99, R1-R9.

Mignatti P, Tsuboi R, Robbins E and Rifkin DB (1989) In vitro angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. J Cell Biol: 108, 671-682.

Minchenko A, Bauer T, Salceda S and Caro J (1994) Hypoxic stimulation of vascular endothelial growth factor expression in vivo and in vitro. Lab Invest: 71, 374-379.

Modlich U, Kaup F, and Augustin HG (1996) Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involves endothelial cell detachment and vessel occlusion. Lab Invest: 41, 771-780.

Monacci W, Merrill M and Oldfield D (1993) Expression of vascular permeability factor/ vascular endothelial growth factor in normal rat tissues. Am J Physiol: 264, 995-1002.

Monaghan P, Robertson D, Amos AS, Dyer MJS, Mason DY and Greaves MF (1992) Ultrastructural localisation of bcl-2 protein. J Histochem Cytochem: 40, 1819-1825.

Montesano R, Vassalli JD, Baird A, Guillemin R and Orci L (1986) Basic fibroblast growth factor induces angiogenesis in vitro. Proc Nat Acad Sci USA: 83, 7297-7301.

Moscatelli D, Presta M and Rifkin DB (1986) Purification of a factor from human placenta that stimulates capillary endothelial cell proteases production, DNA synthesis and migration. Proc Nat Acad Sci USA: 83, 2091-2095.

Moscatelli D, Joseph-Silverstein J, Presta M and Rifkin B (1988) Multiple forms of an angiogenesis factor: basic fibroblast growth factor. Biochimie: 70, 83-87.

Motro B, Itin A, Sachs L and Keshet E (1990) Pattern of interleukin -6 gene expression in vivo suggests a role for this cytokine in angiogenesis. Proc Nat Acad Sci USA: 87, 3092-3096.

Nakashima E, Mukaida N, Kubara Y, Kubota Y, Kono K, Yasumoto K, Ichimura F, Nakanishi I, Miyasaka M and Matsushima K (1995) Human MCAF gene transfer enhances the metastatic capacity of a mouse cachectic adenocarcinoma line in vivo. Pharmacol Res: 12, 1598-1604.

Nett TM , McClellan MC, Niswender GD (1976) Effects of prostaglandins on the ovine corpus luteum: blood flow, secretion of progesterone and morphology. Biol Reprod: 15, 66-78.

Neulen J, Yan Z, Raczec S, Weindel K, Keck C, Weich HA, Marme D and Breckwoldt Al (1995) Human chorionic gonadotropin-dependent expression of vascular endothelial growth factor/ vascular permeability factor in human granulosa cells: Importance in ovarian hyperstimulation syndrome. J Clin Endocrinol Metabol: 80, 1967-1971.

Nguyen M, Millar DG, Yong VW, Korsmeyer SJ and Shore GC (1993) Targeting of bcl-2 to the mitochondrial outer membrane by a COOH terminal signal anchor sequence. J Biol Chem: 268, 25265-25268.

Nicosia SV, Diaz J, Nicosia RF, Saunders BO and Murocacho C (1995) Cell proliferation and apoptosis during development and ageing of the rabbit corpus luteum. Ann Clin Lab Sci: 21, 143-157.

Nissen F (1886) Uber das verhalten der Kerne in den Milchdrusenzellen bei der Absonderung. Arch Mikroscop Anat: 26, 337-342.

Niswender GD and Nett TM (1988) The corpus luteum and its control. In: Knobil E, Neill J, Ewing LL, Greenwald GS, Markert CL, Pfaff DW (Eds) The Physiology of Reproduction. New York, Raven Press, 489-525.

Niswender GD, Reimers TJ, Diekman MA, Nett TM (1976) Blood flow: a mediator of ovarian function. Biol Reprod: 14, 64-81.

Nunez G, and Clarke MF (1994) The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol: 4, 399-403.

O'Shea JD, Nightingale MG and Chamley WA (1977) Changes in small blood vessels during cyclical luteal regression in sheep. Biol Reprod: 17, 162-177.

O'Shea JD, Cran DG and Hay MF (1980) Fate of the theca interna following ovulation in the ewe. Cell Tissue Res: 210, 305-309.

O'Shea JD, Rodgers RJ and Wright PJ (1986) Cellular composition of the sheep corpus luteum in the mid and late luteal phases of the oestrous cycle. J Reprod Fertil :76, 685-691.

Ohara A, Mori T, Taii S, Ban C and Narimoto K (1987) Functional differentiation in steroidogenesis of two cell types of luteal cells isolated from mature human corpora lutea of menstrual cycle. J Clin Endocrinol Metabol: 65, 1192-1200.

Oltvai ZN, Milliman CL and Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell: 74, 609-619.

Osborne BA and Schwartz LM (1994) Essential genes that regulate apoptosis. Trends Cell Biol: 4, 394-399.

Otsuki Y, Misaki O, Sugimoto O, Ito Y, Tsujimoto Y and Akao Y (1994) Cyclic bcl-2 expression in human uterine endometrium during menstrual cycle. Lancet: 344, 28-29.

Park K, Keller G-A and Ferrara N (1993) The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix- bound VEGF. Mol Biol Cell: 4, 1317-1326.

Patwardhan VV and Lanthier A (1985) Luteal phase variations in endogenous concentrations of prostaglandins PGE and PGF and the capacity for their in vitro formation in the corpus luteum. Prostaglandins: 30, 91-98.

Pederson ES (1951) Histogenesis of lutein tissue in the albino rat. Am J Anat: 88, 397-427.

Peitsch MC, Mannherz HG and Tschopp J (1994) The apoptosis endonucleases: cleaning up after cell death? Trends Cell Biol: 4, 37-41.

Peperell JR, Wolcott K and Behrman HR (1992) Effects of neutrophils in rat luteal cells. Endocrinology: 130, 1001-1008.

Pepper MS, Ferrara N, Orci L and Montesano R (1992) Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem Biophys Res Commun: 189, 824-831.

Pesce M and De Felicici M (1994) Apoptosis in mouse primordial germ cells: a study by transmission and scanning electron microscopoe. Anat Embryol: 189, 435-440.

Peters KG, de Vries C and Williams LT (1993) Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. Proc Nat Acad Sci USA: 90, 8915-8919.

Pharris BB, Wyngarden LJ (1969) The effect of prostaglandin F2alpha on the progestogen content of ovaries from pseudopregnant rats. Proc Soc Exp Biol Med: 130, 92-94.

Phillips HS, Hains J, Leung DW and Ferrara N (1990) Vascular endothelial growth factor is expressed in rat corpus luteum. Endocrinology: 127, 965-968.

Powell WS, Hammarstrom S and Samuelsson B (1975) Occurrence and properties of a PGF2α receptor in human corpora lutea. Eur J Biochem: 61, 605-611.

Prenant LA (1898) La valeur morphologique du corps jaune. Son action physologique et theraputique possible. Rev Gen Sci Pures Appl: 9, 646- 652.

Quarto N, Finger FP and Rifkin DB (1991) The NH-2 terminal extension of high molecular weight bFGF is a nuclear targetting signal. J Cell Physiol: 147, 311-318.

Quinn T, Peters KG, deVries C, Ferrara N and Williams LT (1993) Fetal liver kinase-1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Nat Acad Sci USA: 90, 7533-7537.

Raff, MC (1992) Social controls on cell survival and cell death. Nature: 356, 397-340.

Ratts VS, Flaws JA, Kolp R, Sorenson CM and JL Tilly (1995) Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. Endocrinology: 136, 3665-3668.

Ravindranath N, Little-Ihrig LL and Zeleznik AJ (1992a) Characterisation of the levels of messenger ribonucleic acid that encode for luteinising hormone receptor during the luteal phase of the primate menstrual cycle. J Clin Endocrinol Metabol: 74, 779-785.

Ravindranath N, Little-Ihrig L, Phillips H, Ferrara N and Zeleznik AJ (1992b) Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. Endocrinology: 131, 254-260.

Redmer DA and Reynolds LP (1996) Angiogenesis in the ovary. Rev Reprod: 1, 182-192.

Redmer DA, Dai Y, Li J, Charnock-Jones DS, Smith SK, Reynolds LP and Moor RM (1996) Characterisation and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. J Reprod Fertil: 108, 157-165.

Redmer DA, Rone JD and Goodman AL (1985) Evidence for a non-steroidal angiotropic factor from the primate corpus luteum: stimulation of endothelial cell migration in vitro. Proc Soc Exp Biol Med: 179, 136-140.

Redmer DA, Grazul AT, Kirsch JD and Reynolds LP (1988) Angiogenic activity of bovine corpora lutea at several stages of luteal development. J Reprod Fertil: 82, 627-634.

Reed JC (1994) Bcl-2 and the regulation of programmed cell death. J Cell Biol: 124, 1-6.

Renko M, Quarto N, Morimoto T and Rifkin DB (1991) Nuclear and cytoplasmic localisation of different basic fibroblastic growth factor species. J Cell Physiol: 109, 1-40.

Reynolds LP (1986) Utero-ovarian interactions during early pregnancy: role of conceptus induced vasodilation. J Anim Sci: 62 (suppl2), 47-61.

Reynolds LP, Killilea SD and Redmer DA (1992) Angiogenesis in the female reproductive system. FASEB J: 6, 886-892.

Reynolds LP, Killilea SD, Grazul-Bilska AT and Redmer DA (1994) Mitogenic factors of corpora lutea. Prog Growth Factor Res: 5, 159-175.

Richards JS (1980) Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell development. Physiol Rev: 60, 51-89.

Ricke WA, Redmer DA and Reynolds LP (1995) Initial characterisation of mitogenic factors produced by porcine corpora lutea throughout the estrous cycle. Biol Reprod: 52 (Supp 1), 112.

Rifkin DB and Moscatelli D (1989) Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol: 109, 1-6.

Riparbelli MG, Callalaini G, Tripoldi SA, Cintorino M, Tosi P and Dallai R (1995) Localisation of the bcl-2 protein to the outer mitochondrial membrane by electron microscopy. Exp Cell Res: 221, 363-369.

Roberts WG and Palade GE (1995) Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. J Cell Sci: 108, 2369-2379.

Roby KF and Terranova PF (1989) Localisation of tumour necrosis factor (TNF) in rat and bovine ovary using immunohistochemistry and cell blot: evidence for granulosal production. In: Hirshfield AN (Ed) Growth factors and the Ovary. New York, Plenum Press, 273-278.

Roby KF Lyles WR and Terranova PF (1990) Immunological evidence for a human ovarian tumour necrosis factor alpha. J Clin Endocrinol Metabol: 71, 1096-1102.

Rodger FE, Fraser HM, Young FM and Illingworth PJ (1997) Endothelial cell proliferation is triggered by the LH surge but not hCG stimulation in the human corpus luteum. Hum Reprod: 12, 1723-1729.

Rojas FJ, Moretti-Rojas IM, Balmaceda JP and Asch RH (1989) Changes in adenylate cyclase activity of the human and the non-human primate corpus luteum during the menstrual cycle and pregnancy. J Clin Endocrinol Metabol: 68, 379-385.

Rothchild I (1981) The regulation of the mammalian corpus luteum. Rec Prog Hormone Res; 37, 183-298.

Rueda B, Wegner JA, Marion SL, Wahlen DD and Hoyer P (1995) Internucleosomal DNA fragmentation in ovine luteal tissues associated with in vivo and in vitro analyses. Biol Reprod: 52, 305-312.

Rueda BR, Tilly Ki, Botros IW, Jolly PD, Hansen TR, Hoyer PB and Tilly JL (1997) Increased Bax and interleukin-1 converting enzyme messenger ribonucleic acid levels coincide with apoptosis in the bovine corpus luteum during structural regression. Biol Reprod: 56, 186-193.

Salim A, Zalud I, Farmakides G, Sohulman H, Kurjak A and Latin V (1994) Corpus luteum blood flow in normal and abnormal pregnancy- evaluation with transvaginal colour and pulsed Doppler ultrasonography. J Ultrasound Med: 13, 971-975.

Sasaki K, Murakami R, Kawasaki M and Takahashi M (1987) The cell cycle associated change of Ki-67 reactive nuclear antigen expression. J Cell Physiol: 133, 579-584.

Sasaki K, Murakami R, Tsuji T, Shinozaki F and Takahashi M (1988) Relationship between labelling indices of Ki67 and BrdUrd in human malignant tumours. Cancer: 62, 989-993.

Sasano H, Okamoto M, Mason JI, Simpson ER, Mendelson CR, Sasano N and Silverberg SG (1989) Immunolocalisation of aromatase, 17-alpha hydroxylase and side-chain cleavage cytochromes P450 in the human ovary. J Reprod Fertil: 85, 163-169.

Saunders JW and Fallon JF (1967) Cell death in morphogenesis. Major Problems in Developmental Biology. New York, Academic Press, 289-314.

Savill JS, Dransfiled L, Hogg N and Haslett C (1990) Vitronectin receptor mediated phagocytosis of cells undergoing apoptosis. Nature: 343, 170-173.

Sawyer HR, Niswender KD, Braden TD and Niswender GD (1990) Nuclear changes in ovine luteal cells in response to PGF2α. Dom An Endocrinol: 7, 229-238.

Schams D, Amselgruber W, Einspanier R, Sinowatz F and Gospodarowicz D (1994) Localisation and tissue concentration of basic fibroblast growth factor in the bovine corpus luteum. Endocrine J: 2, 907-912.

Schweiki D, Itin A, Soffer D and Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediated hypoxia-initiated angiogenesis. Nature: 359, 843-845.

Schweiki D, Itin A, Neufeld G, Gitay-Goren H and Keshet E (1993) Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest: 91, 2235-2243.

Schwinzer R. (1989) Cluster report: CD45/CD45R. In: Knapp W (Ed) Leucocyte typing IV. White cell differentiation antigens. Oxford, UK, Oxford University Press, 628-634.

Segaloff DL, Wang H and Richards JS (1990) Hormonal regulation of luteinising hormone/ chorionic gonadotrophin receptor mRNA in rat ovarian cells during follicular develoment and luteinisation. Mol Endocrinol: 4, 1856-1865.

Senger D, Connolly D, Peruzzi CA, Feder J and Dvorak HF (1987) Purification of vascular permeability factor (VPF) from tumour conditioned medium. Fed Proc: 46, 2102-2104.

Senger DR, Connolly DT, Van de Water L, Feder J and Dvorak HF (1990) Purification and NH-2 terminal amino acid sequence of guinea pig tumour secreted vascular permeability factor. Cancer Res: 50, 1774-1778.

Senger DR, Galli SJ, Dvorak AM, Peruzzi CA, Harvey VS and Dvorak HF (1993) Tumour cells secrete a vascular permeability factor that promoted accumulation of ascites fluid. Science: 219, 983-985.

Server AC and Mobley WC (1991) Neuronal cell death and the role of apoptosis: the molecular basis of cell death. In: Tomei LD and Cope FO (Eds) New York, Cold Spring Harbour Laboratory Press, 47-60.

Shalaby F, Rossant J, Yamaguchi TP, Gertenstein M, Wu XF, Breitman MC and Schuh AC (1995) Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. Nature: 376, 62-66.

Sharkey AM, Charnock-Jones DS, Boocock CA, Brown KD and Smith SK (1993) Expression of mRNA for vascular endothelial growth factor in human placenta. Journal of Reproduction and Fertility: 99, 609- 615.

Sharp DC, McDowell KJ, Weitenhauer J and Thatcher WW (1989) The continuum of events leading to maternal recognition of pregnancy in mares. J Reprod Fertil: (suppl) 37, 101-107.

Shi S, Chaiwun B, Young L, Cote RJ and Taylor CR (1993) Antigen retrieval technique utilising citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. Histochem Cytochem: 41, 1599-1604.

Shibuya M, Yamaguchi S and Yamane A (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase (flt) closely related to the fms family. Oncogene: 8, 519-527.

Shifren JL, Doldi N, Ferrara N, Mesiano S and Jaffe RB (1994) In the human fetus, vascular endothelial growth factor is expressed in epithelial cells and myocytes but not vascular endothelium: Implications for a mode of action. J Clin Endocrinol Metabol: 79, 316-322.

Shikone T, Yamoto M, Kokawa K, Yamashita K, Nishimori K and Nakano R (1996) Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. J Clin Endocrinol Metabol: 81, 2376-2380.

Shima DT, Adamis AP, Ferrara N, Yeo KT, Yoe TK, Allende R, Folkman J and D'Amore PA (1995) Hypoxic induction of vascular endothelial growth factors in

the retina: identification and characterisation of vascular endothelial growth factor (VEGF) as the sole mitogen. Mol Med: 2, 64-71.

Shiraishi S, Nakagava K, Kinukawa N and Sueshi K (1996) Immunohistochemical localisation of vascular endothelial growth factor in the human placenta. Placenta: 17, 111-121.

Simon C, Frances A, Piquette G and Polan ML (1994) Immunohistochemical localisation of the interleukin-1 sytem in the mouse ovary during follicular growth, ovulation and luteinisation. Biol Reprod: 50, 449-457.

Simpson JL (1996) Fetal Wastage. In: Gabbe SG, Niebyl JR, Simpson JL (Eds) Obstetrics: normal and abnormal pregnancies. New York, Churchill Livingstone, 181-200.

Sjorgen A, Holmes PV and Hillensjo T (1991) Interleukin 1 alpha modulated luteinising hormone stimulated cyclic AMP and progesterone release from human granulosa cells in vitro. Hum Reprod: 6, 910-913.

Smith PE (1930) Hypophysectomy and a replacement therapy in the rat. Am J Anat: 45, 205-273.

Stavri GT, Zachary IC, Baskerville PA, Martin JF and Erusalimsky JD (1995) Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells: Synergistic interaction with hypoxia. Circulation: 92, 11-14.

Stocco DM and Clark BJ (1996) Role of the steroid acute regulatory protein (STAR) in steroidogenesis. Biochem Pharmacol: 51, 197-205.

Stouffer RL, Nixon WE, Gulyas BJ and Hodgen GD (1977) Gonadotrophin sensitive progesterone production by rhesus monkey luteal cells in vitro: a function of age of the corpus luteum during the menstrual cycle. Endocrinology: 100, 506-512.

Stouffer RL, Ottobre JS and Vandevort CA (1987) Regulation of the primate corpus luteum during early pregnancy In: Stouffer RL (Ed) The primate ovary. New York, Plenum Press, 207-220.

Strauss JF, Schuler LA, Rosenblum LF and Tanaka T (1981) Cholesterol metabolism by ovarian tissue. Lipid Res: 18, 99-157.

Strott CA, Yoshimi T, Ross GT and Lipsett MB (1969) Ovarian Physiology: relationship between plasma LH and steroidogeneisis by the follicle and corpus luteum; effect of hCG. J Clin Endocrinol Metabol: 29, 1157-1167.

Swanston I, McNatty KP and Baird DT (1977) Concentration of prostaglandin F2α and steroids in the human corpus luteum. J Endocrinol: 73, 115-122.

Tabibzadeh S, Kong OF, Satyaswaroop PG , Kang QF and Aktories K (1994) Distinct regional and menstrual cycle dependent distribution of apoptosis in human endometrium-potential regulatory role of T-cells. Endocrine J:2, 87-95.

Talavera F and Menon KM (1989) Regulation of rat luteal cell high density lipoprotein receptors: up regulation in response to changes in intracellular cholesterol concentration. Endocrinology: 125, 2015-2021.

Talwar GP (1997) Fertilily regulating and immunotherapeutic vaccines reaching human trials stage. Hum Reprod Update: 3, 301-310.

Tan GJS, Tweedale R and Biggs JSG (1982) Oxytocin may play a role in the control of the human corpus luteum. J Endocrinol: 95, 65-70.

Tanaka S, Saito K and Reed JC (1993) Structure function analysis of the bcl-2 oncoprotein- addition of a heterologous transmembrane domain to portions of the bcl-2 beta protein restores function as a regulator of cell survival. J Biol Chem: 268, 10920-10926.

Tanaka S, Shimoya Y, Hamamatsu M and Hashimoto M (1983) Binding sites for PGF2α in human corpora lutea. Asia-Oceania J Obstet Gynaecol: 9, 445-451.

Tandeski TR, Juengel J, Nett TM and Niswender GP (1996) Regulation of messenger RNA encoding LDL receptor and HDL binding protein in ovine corpora lutea. Rep Fertil Devel: 8, 107-114.

Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL and Shows TB (1991) Identification of a new endothelial growth factor receptor tyrosine kinase. Oncogene: 6, 519-524.

Terman BI, Vermazzen MD, Carron ME, Dimitrov D, Armellino DC, Gospodarowicz D and Bohlen P (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. Biochem Biophys Res Commun: 34, 1578-1586.

Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. Science: 267, 1456-1462.

Tilly JL (1993) Ovarian follicular atresia: a model to study the mechanisms of physiological cell death. Endocrine J: 1, 67-72.

Tilly JL (1996) Apoptosis and ovarian function. Rev Reprod: 1, 162-172.

Tilly JL and Ratts VS (1996) Biological and clinical importance of ovarian cell death. Contemp Obstet Gynaecol: 41, 59-86.

Tilly JL and Tilly KI (1995) Inhibitors of oxidative stress mimic the ability of follicle stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology: 136, 242-252.

Tilly JL, Tilly KI, Kenton ML and Johnson AL (1995) Expression of members of the bcl-2 gene family in the immature rat ovary: Equine chorionic gonadotrophin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. Endocrinology: 136, 232-241.

Tischer E, Mitchell R, Hartman T, et al. (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem: 266, 11947-11954.

Toi, M (1995) Endothelial growth factors: a target for anti-angiogenesis. Cancer J: 8, 315-319.

Tovanabutra S, Illingworth PJ, Ledger WL, and Baird DT (1993) The relationship between peripheral immunoactive inhibin, human chorionic gonadotrophin, oestradiol and progesterone during human pregnancy. Clin Endocrinol: 38, 101-107.

Townson DH, Naftalin DM, Flory CM, Warren JS and Keyes PL (1995) Localisation of monocyte chemoattractant protein-1 (MCP-1) in corpora lutea of the rat ovary. Biol Reprod: 52, Supplement 1, 152.

Townson DH, Warren JS, Flory CM, Natfalin DM and Keyes PL (1996). Expression of monocyte chemoattractant protein-1 in the corpus luteum of the rat. Biol Reprod: 54, 513-520.

Tsujimoto Y and Croce CM (1986) Analysis of the structure, transcripts and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Nat Acad Sci USA: 83, 5214-5218.

Ueda N and Shah SV (1994) Apoptosis. J Lab Clin Med: 124, 169-177.

Vande-Weil RL, Bogumil J, Dyrenfurth I , Ferin M, Jenelewicz R, Warren M Rizkallah T and Mikhail G (1970) Mechanisms regulating the menstrual cycle in women. Rec Prog Hormone Res: 26, 63-95.

Vassali P (1992) The pathophysiology of tumour necrosis factors. Ann Rev Immunol: 10, 411-452.

Vaux DL, Cory S and Adams J (1988) Bcl-2 gene promotes haemopoetic cell survival and cooperates with c-myc to immortalise pre-B cells. Nature: 325, 440-442.

Wang F, Riley JCM and Behrman HR (1993) Immunosuppressive levels of glucocorticoid block extrauterine luteolysins in the rat. Biol Reprod: 49, 66-73.

Wang L, Robertson S, Seamark RF and Norman RJ (1991) Lymphokines, including interleukin-2, alter gonadotropin stimulated progesterone production and proliferation of human granulosa lutein cells. J Clin Endocrinol Metabol: 72, 824-831.

Wang LJ, Pasco V, Petrucco OM and Norman RJ (1992) Distribution of leukocyte subpopulations in the human corpus luteum. Hum Reprod: 7, 197-202.

Wang TTY and Phang JM (1995) Effects of estrogen on apoptotic pathways in human breast cell cancer line MCF-7. Cancer Res: 55, 2487-2489.

Wentz AC and Jones GS (1973) Transient luteolytic effect of prostaglandin F2α in the human. Obstet Gynaecol: 42, 172-181.

Westfahl PK and Resko JA (1983) Effects of clomiphene on luteal function in the non-pregnant cynomolgus macaque. Biol Reprod: 29, 963-969.

Wheeler T, Elcock CL and Anthony FW (1995) Angiogenesis and the placental environment. Placenta: 16, 289-296.

Wilks JW (1980) Inhibition of the monkey corpus luteum with 15-methyl prostaglandins. Prostaglandins: 30, 739- 805.

Williams GT, Smith CA, Spooncer E, Dexter TM and Taylor DR (1990) Haemopoetic colony stimulating factors promote cell survival by suppressing apoptosis. Nature: 343, 76-79.

Wiltbank MC, Dysko RC, Gallagher KP and Keyes PL (1988) Relationship between blood flow and steroidogenesis in the rabbit corpus luteum. J Reprod Fertil: 84, 213-220.

Wiltbank MC, Gallagher KP, Dysko RC, and Keyes PL (1989) Regulation of blood flow to the rabbit corpus luteum: effects of estradiol and human chorionic gonadotrophin. Endocrinology: 124, 605-611.

Wyllie AH (1992) Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: An overview. Cancer Metast Rev: 11, 95-103.

Wyllie AH (1993) Apoptosis. Br J Cancer: 67, 205-208.

Wyllie AH, Kerr JFR and Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol: 68, 251-306.

Yamaji T, Dierscheke DJ, Bhattacharya AN and Knobil E (1972) The negative feedback control by oestradiol and progesterone of LH secretion in the ovarectomised rhesus monkeys. Endocrinology: 90, 771-777.

Yan Z, Hunter V, Weed J, Hutchison S, Lyles R and Terranova PF (1993) Tumour necrosis factor alpha alters steroidogenesis and stimulates proliferation of human ovarian granulosa cells in vitro. Fertil Steril: 57, 332-338.

Yin XM, Oltvai ZN and Korsmeyer SJ (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature: 369, 321-323.

Yoshimura T, Yuhki N, Moore SK, Apella E, Lehrman MI and Leonard EJ (1989). Human monocyte chemoattractant protein-1 (MCP-1). Full length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. FEBS Let: 244, 487-493.

Young FM, Rodger FE, Cowen G, Woad K, Lunn S, Illingworth PJ and Fraser HM (1996) Localisation of apoptosis after induced luteal regression in the primate. Biol Reprod: 54 (Suppl 1), Abstract 359.

Zagzag D (1995) Angiogenic growth factors in neural embryogenesis and neoplasia. Am J Pathol: 146, 293-298.

Zeleznik AJ (1991) Control of luteal endocrine function. In: Hillier SG (Ed.) Ovarian Endocrinology. Boston, Blackwell Scientific Publications, 167-189.

Zeleznik AJ, Ihrig LL and Bassett SG (1989) Developmental expression of Ca++/Mg++ dependent endonuclease activity in rat granulosa and luteal cells. Endocrinology: 125, 2218-2220.

Zheng J, Redmer DA, and Reynolds LP (1993) Vascular development and heparin-binding growth factors in the bovine corpus luteum at several stages of the estrous cycle. Biol Reprod: 49, 1177-1189.

Zheng J, Fricke PM, Reynolds LP and Redmer DA (1994) Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. Biol Reprod: 51, 623-632.

Zhu X, Koyima H, Chirino A et al. (1991) Three dimensional structure of acidic and basic fibroblast growth factor. Science: 251, 90-93.